University of Alberta

Activated States of Nicotinic Acetylcholine Receptor

by

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ABSTRACT

Single channel currents were investigated using *Torpedo* nicotinic acetylcholine receptors reconstituted in giant liposomes. Three current populations were found: two full-conductance currents differentiated by open duration and a subconductance current. The frequencies of subconductance and full-conductance currents were sigmoidal functions of log acetylcholine (ACh) concentration. EC50 values for the subconductance and combined full-conductance curves differed by a factor of 7. The frequency of long-duration full-conductance openings was concentration-dependent. Currents elicited by agonists with a semi-rigid structure produced currents of the same amplitudes. The frequency-concentration curve for subconductance openings evoked by 1,1-dimethyl-4-phenylpiperazinium was superimposable on that for ACh, but the full conductance concentration-frequency curve was shifted ~2.5 orders of magnitude rightward. The separation of subconductance and full-conductance current-frequency curves was interpreted to indicate independent activation sites. A novel bis-functional semi-rigid compound elicited a fourth activated state, a long-duration subconductance opening, which requires further investigation.

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LIST OF ABBREVIATIONS

ACh	acetylcholine
ANOVA	analysis of variance
A-BgTx	alpha bungarotoxin
AChBP	acetylcholine binding protein
AChE	acetylcholinesterase
BrACh	bromoacetylcholine
BSA	bovine serum albumin
CCh	carbamylcholine
CHAPS	3-[3-cholamidopropyl)-dimethylammonio]-1-1propane sulfonate
DK1	bis(N,N-dimethylpiperidinyl) oxalate
DMPP	1,1-dimethyl-4-phenylpiperazinium
dTC	d-tubocurarine
DTT	dithiothreitol
EC_{50}	half-maximal concentration required for channel activation
EDTA	ethylenediaminetetraacetic acid
GABA	gamma-aminobutyric acid
$5\text{-}HT_3$	5-hydroxytryptamine
HEPES	4-(hydroxyethyl)-1-piperazinethanesulfonic acid
IAS	5-iodoacetamidosalicylic acid
IANBD	4-([iodoacetoxy)ethyl]methylamino)-7-nitro-2,1,3-benzoxadiazole
K_D	equilibrium ligand dissociation constant
LGIC	ligand-gated ion channel
MBTA	4-(N-maleimido)benzyltrimethyl ammonium
nAChR	nicotinic acetylcholine receptor
P2X	purinergic ATP receptor
PMSF	phenylmethylsufonyl fluoride
PTMA	phenyltrimethylammonium
SD	standard deviation
SEM	standard error of the mean
SubCh	suberyldicholine

CHAPTER 1

Introduction

OVERVIEW OF THE NICOTINIC ACETYLCHOLINE RECEPTOR

Nicotinic acetylcholine receptors (nAChRs) play an important role in excitatory synaptic transmission in both the central nervous system and at the neuromuscular junction. The nAChR belongs to the superfamily of cys-cys loop ligand gated ion channels (LGICs) (Lindstrom *et al.*, 1987; Stroud *et al.*, 1990) that also includes 5-hydroxytryptamine ($5HT_3$) (Maricq *et al.*, 1991; Miyake *et al.*, 1995), γ-aminobutyric acid ($GABA_A$) (Schofield *et al.*, 1987) and glycine receptors (Grenningloh *et al.*, 1987a; Grenningloh *et al.*, 1987b). Generally, ligand gated ion channels possess a neurotransmitter binding site or sites, which, upon occupation, activate the receptor. Activation of the receptor opens the channel, allowing ions to flow across the cell membrane along its electrochemical gradient. This flow of current may result in the depolarization of the cell if there is a net flow of positive ions inward (or if there is a net flow of negative ions outward) which leads to an increase in cellular excitability. A net flow of positive ions outward (or a net flow of negative ions inward) results in the hyperpolarization of the cell membrane and decrease in excitability of the cell.

Members of the LGIC superfamily share significant similarities in both structure and function. Therefore, it is possible to understand many characteristics of the superfamily through the study of just one of its members. The nAChR was used as the model for the rest of LGIC superfamily as the *Torpedo* electroplax contains a highly concentrated source of receptor ($\sim 3 \times 10^4$ receptors/μm^2; Bourgeois *et al.*, 1972), the receptors of which are highly homologous to nicotinic receptors present in the neuromuscular junctions of mammals (Conti-Tronconi & Raftery, 1982). The efficient purification and characterization of the nAChR (Moore & Brady, 1977; Elliott *et al.*, 1980; Kemp *et al.*, 1980; Conti-Tronconi & Raftery, 1982; Gotti *et al.*, 1982) has been enabled through the use of snake α-neurotoxins which bind with high affinity and specificity to the receptor (reviewed by Lee, 1972). The abundance of the receptor in *Torpedo* electroplax and the relative ease of purification allowed many insights to be gained into the biochemical and functional properties before molecular biological techniques would allow the expression of other LGICs in quantities sufficient for study.

Peripheral, or muscle-type nAChRs, are predominantly located at the endplate of the neuromuscular junction and, upon activation, result in the depolarization of muscle fibres and subsequently muscle contraction. The sequence of events that leads to postsynaptic excitation begins with the depolarization of the motor nerve terminal and the release of ACh stored within vesicles into the synaptic cleft. The concentration of ACh within the cleft varies dependent on the number of synaptic vesicles released, however it generally ranges from 100 μM to 1 mM (Kuffler & Yoshikami, 1975; Katz & Miledi, 1977). It only takes 6 μs for the ACh to diffuse across the synaptic cleft and bind to nAChR on the postsynaptic membrane (Edmonds *et al.*, 1995). When ACh binds to the receptor, it induces a conformational change and allows the channel to enter the open state. From noise analysis, Katz and Miledi (1970, 1971) predicted that the receptor remains open for less than 1 ms during which it becomes permeable to cations, the most important of which is Na^+. During the short open period, sodium enters the postsynaptic cell (~10^7 ions/s) causing depolarization and muscle contraction. The concentration of ACh within the synaptic cleft drops rapidly as ACh is hydrolyzed by acetylcholinesterase or ACh diffuses out of the synaptic cleft (Katz & Miledi, 1977).

If, however, the concentration of ACh remains high within the cleft, the constant exposure to agonist results in the receptor undergoing conformational changes which lead to desensitization (Ochoa *et al.*, 1989). In the desensitized state, the receptor remains closed despite agonist binding and will not revert to a state in which it can be activated until all agonist is removed. The desensitization process has at least two components – a rapid desensitization phase which occurs over the timescale of seconds and a slow desensitization phase which occurs over the time scale of minutes (Feltz & Trautmann, 1980), although kinetic analysis from single channel currents reveals that there may be up to five components to desensitization (Elenes & Auerbach, 2002). Further complicating matters, the desensitized state has a higher affinity for ACh (K_d~10 nM) than the resting state of the receptor (EC_{50}~100 μM) (Raftery *et al.*, 1983) which suggests that prolonged exposure to very low concentrations of ACh may result in desensitization of many receptors at the muscle endplate. Desensitization may not play a large role in normal neuromuscular transmission; its effects, however, must be accounted for in experiments in which the receptor is exposed to high concentrations of agonist for long periods of time.

OVERVIEW OF THE STRUCTURE OF THE nAChR

Transmembrane topology and subunit composition

The nicotinic acetylcholine receptor is a 270 kD protein composed of four homologous subunits arranged around a central cation pore with the stoichiometry $\alpha_2\beta\gamma\delta$. Early studies of the *Torpedo* nAChR revealed four distinct subunits, however the stoichiometry and arrangement of the subunits were a matter of intense debate. The N-terminal amino acid sequencing of all subunits by Raftery *et al.* (1980) demonstrated the now accepted nAChR structure of $\alpha_2\beta\gamma\delta$ and also revealed that the subunits had homologous amino acid sequences. The sequencing of the four subunits revealed a large extracellular N-terminal domain of approximately 210 amino acids which contain the agonist binding sites, four transmembrane domains termed M1-M4, and a short extracellular C-terminus (Noda *et al.*, 1983; Changeux *et al.*, 1992a; Karlin, 1993; Karlin & Akabas, 1995). A large intracellular domain between M3-M4 contains phosphorylation sites for numerous protein kinases including cAMP-dependent protein kinase and protein kinase C. Phosphorylation of the receptor may enhance the desensitization rate of the receptor (Huganir *et al.*, 1986; Miles *et al.*, 1987).

Elucidation of the arrangement of the subunits within the receptor excluded a number of possibilities including adjacent α-subunits (Schiebler *et al.*, 1980), an α-β-α arrangement (Wise *et al.*, 1981) or α-δ-α (Karlin *et al.*, 1983) which led Karlin *et al.* (1983) to propose an α-γ-α-δ-β arrangement. Expression studies found that the α-subunit would associate with either the γ- or δ-subunits, but not the β-subunit which further entrenched the theory that the α-subunits (and the neurotransmitter binding sites) were associated with the γ- and δ-subunits (Blount *et al.*, 1990). Photoaffinity labeling with [^{3}H]d-tubocurarine provided further evidence for the association of the α- and γ/δ-subunits. Pedersen and Cohen (1990) found that the photolabel covalently labelled the two α-subunits as well as both the γ- and δ-subunits with affinities similar to that previously reported for the high and low affinity binding sites. From the previous evidence, it was suggested that the α-γ-α-δ-β arrangement was indeed correct, however, at the time, there was still debate as to whether the arrangement was either clockwise or counter-clockwise when viewed from the synaptic cleft (Hucho *et al.*, 1996; Arias, 2000).

Three-dimensional structure of the nAChR and channel gating

The three-dimensional structure of the *Torpedo* nAChR has been largely elucidated by the efforts of Unwin and his group using cryo-electron microscopy techniques (Unwin *et al.*, 1988; Unwin, 1993; Unwin, 1995; Unwin *et al.*, 2002; Miyazawa *et al.*, 2003; Unwin, 2003; Unwin, 2005). Isolated membranes from electrocytes convert readily to long tubular crystals containing nAChRs which have allowed Unwin *et al.* to examine the receptor in immense detail without crystallizing the protein required for x-ray diffraction.

The general dimensions of the receptor reported by Unwin (1993) at a resolution of 9Å were little different from what was previously reported (Toyoshima & Unwin, 1990) although the higher resolution allowed for the discernment of fine structures and cavities on the surface of the protein. The nAChR is 80Å in diameter and 125Å long. The protein projects 60Å into the synaptic cleft and extends 20Å into the cytoplasm on the opposite end. Approximately 30Å above the lipid bilayer Unwin found three density peaks on each subunit. These peaks, formed by rods that he presumed were α-helices, formed cavities in the center of the α-subunits which he suggested formed the agonist binding sites. These cavities were not as pronounced on the homologous subunits nor were they present at the α/γ and α/δ interfaces as had been previously hypothesized (Karlin, 1993). Each subunit contributed a rod, most likely an α-helix, that formed a kink at its mid-point. This kink in the M2 region may form the gate which controls the permeability of the pore to cations. Unwin hypothesized that the gate may be made by the projection of the side-chains contributed by the highly conserved αLeu251 residue at the level of the kink which would create a hydrophobic barrier and thus prevent ions from passing through the channel.

All previous images by Unwin consisted of receptors in the closed conformation. To determine the structural changes related to agonist binding, Unwin (1995) exposed nAChR tubes to ACh and then rapidly froze them within 5 ms in an effort to capture receptors in an open conformation. The largest disturbance was detected on the α-subunit adjacent to the δ-subunit (α_δ). The rods of the α_δ-subunit rotated approximately 28° anticlockwise when exposed to ACh, whereas the α_γ subunit rotates to lesser extent and the β-subunit is drawn away from the α_δ-subunit. These rotations are translated to the M2 kink which draws the leucine side chains away from the central pore thus allowing ions to flow through the channel. The exact mechanism is unknown, however the fact remains that agonist binding is translated through the protein resulting in the opening of the channel.

Further refinement of the technique has allowed a resolution of 4.6Å to be achieved (Unwin, 2002; Unwin *et al.*, 2002; Miyazawa *et al.*, 2003, Unwin, 2005). Visualization of the extracellular vestibules reveals numerous openings that may act as a molecular sieve, preventing large cytoplasmic molecules from reaching the vicinity of the pore. The gate formed by a kink in the M2 region appears to have an approximate radius of 3.5Å which is slightly smaller than the radius of a Na^+ or K^+ ion. This would effectively block ions from permeating through the channel when the receptor was in the closed conformation. At lower resolutions, it was not possible to determine how a molecule could enter the binding pockets within the α-subunits but higher resolutions show narrow tunnels which connect the cavities with the extracellular vestibule. Unwin suggested that these tunnels are the most likely pathway for an agonist to bind to the receptor. Once bound, the receptor undergoes a conformational change resulting in the opening of the channel. To achieve this, Unwin postulates a series of events beginning with a rotation of the inner β-sheets which form the binding pocket. This rotation pushes the α-subunits toward their associated subunit (γ or δ) as previously described. Movement of the Cys-loop initiated by the rotation of the subunits may allow it to come into contact with the M2-M3 extracellular loop, which may be transmitted down the M2 helices, removing the kink and opening the channel. Unwin's mechanism provides an elegant pathway through which ligand binding is translated into an open gate.

The crystallized structure of the acetylcholine binding protein (AChBP)

The AChBP is a soluble protein produced by the glial cells of the snail *Lymnaea stagnalis*. The protein is released by glial cells into the synaptic cleft in an ACh-dependent manner where it binds ACh, thus acting as a buffer and preventing the build up of ACh in the synaptic cleft (Smit *et al.*, 2001). Crystallization of the protein allowed characterization at a resolution of 2.7Å (Brejc *et al.*, 2001). The AChBP consists of five identical monomers that assemble to form a homopentamer similar in structure to the nAChR. The height of the protein is 62Å, the diameter is 80Å, and the central pore is approximately 18Å in diameter. These dimensions are similar to those reported by Unwin (1993, 1995) for the *Torpedo* nAChR although the length of the AChBP is much less than that of the nAChR as it does not possess any transmembrane domains. This lack of a transmembrane domain results in the N-terminal domain located on one side of the protein, while the C-terminal domain is located on the other. Each monomer consists of 210 amino acids, the ligand binding domains of which share approximately 20-24% homology with other nicotinic receptors and 15-18% homology with other ligand gated ion channels. The AChBP displays the greatest homology with the homomeric α_7 nAChR as might be expected as they both contain five identical subunits. The pharmacological properties of the AChBP are similar to other nAChRs as they bind typical nicotinic agonists such as ACh and nicotine, as well as competitive antagonists such as d-tubocurarine and α-bungarotoxin. Furthermore, the AChBP contains a Cys-loop similar to others in the LGIC superfamily located on C-terminal end of the protein.

The ligand binding pocket contains all of the amino acids previously identified to be highly conserved among the LGIC superfamily (Brejc *et al.*, 2001; Smit *et al.*, 2001). Without listing all the residues, it is clear that these highly conserved residues are essential for the proper recognition and binding of nicotinic ligands. The authors speculated that the agonist binding pocket was located at the interfaces of the subunits and was flanked by the amino acid residues that form the principle and complementary binding loops which were visualized for the first time with this technique (Figure 1-1). This visualization has allowed the structural modeling of the positions of these binding loops, the positions of which had only been speculated upon (Le Novere *et al.*, 2002; Sine, 2002). The crystallization of the AChBP has provided the most intricate view of the Cys-loop family of receptors, especially the ligand binding domain. With this new knowledge, it may be possible to model the effects of ligand binding and allow the intelligent design of new ligands.

THE LIGAND BINDING SITES OF THE nAChR

Kinetic mechanisms of receptor activation

The nAChR can exist in multiple conformations including the resting state, the active (or open) state, and the desensitized state. Binding of agonist to the receptor greatly increases the likelihood that the receptor will exit the resting state and enter the active state. Numerous kinetic models have been proposed to explain the relatively simple observation that the addition of agonist activates the receptor. The Monod, Wyman, Changeux model (MWC model) (Monod *et al.*, 1965) postulates that multisubunit allosteric proteins exist in equilibrium between two or more conformational states. Once an agonist(s) binds, all subunits of the receptor simultaneously undergo a conformational change resulting in activation of the receptor. In this model, binding of ligand shifts the equilibrium to the state for which the ligand has the higher affinity. Agonists have a higher affinity for the active state and would therefore stabilize the receptor in that state, whereas antagonists have a higher affinity for the closed state therefore shifting the equilibrium towards the closed state.

Koshland, Nemethy and Filmer (KNF model; 1966) proposed an alternate hypothesis of receptor activation in which the receptor remained in the resting state until an agonist molecule was bound. The binding of an agonist induces allosteric conformational changes in the receptor which lead to the activated state. Antagonists, on the other hand, do not induce a conformational change in the receptor upon binding and therefore do not cause channel to open. Both models are useful in describing certain aspects of ligand binding and receptor activation, although both oversimplify the complex process of channel activation and therefore many phenomena are not adequately modeled. These models are, however, useful for conceptualizing channel activation and provide a framework for considering the effects of agonists and agonists on receptors.

Ligand binding sites

Early studies into the location of the ligand binding sites of the nAChR used reactive ligands for both affinity and photoaffinity labeling experiments. The presence of a readily reducible bond between αCys192-αCys193 provided an accessible site for labeling via alkylation by the agonist [^{3}H]bromoacetylcholine (BrACh) (Moore & Raftery, 1979a; Moore & Raftery, 1979b) or the antagonist [^{3}H]4-(N-maleimido) benzyltrimethyl ammonium ([^{3}H]MBTA) (Karlin & Cowburn, 1973). Using these labels, it was found that most of the ligand binding was associated with the α-subunit. Amino acid sequencing determined that the antagonist MBTA only labeled the two cysteine residues (αCys192-αCys193) found on the α-subunit which suggested that these two residues were located near to the ligand binding site (Kao *et al.*, 1984).

Using a variety of alkylating ligands, a number of amino acids on the α-subunit were implicated in ligand binding. The labeled amino acids on the α-subunit (Tyr-190, Cys192/Cys-193, Trp-149, and Tyr-93) were distributed into three regions which led to the three-loop hypothesis (Galzi *et al.*, 1990; Galzi *et al.*, 1991; Changeux *et al.*, 1992b). These three loops (A-C), containing residues from the regions 93, 149-152, and 180-200, form a binding pocket that is similar in structure to that found in acetylcholinesterase (Sussman *et al.*, 1991). The highly conserved aromatic amino acids Tyr-190, Trp-149, Tyr-93 and Tyr-198 are crucial for ACh binding as the π-cation, dipole-cation, H-bonding, and van der Waals interactions stabilize the molecule within the binding pocket.

Further stabilization is provided by amino acids found on the adjacent γ/δ subunits. γTrp-55, δTrp-57, γTyr-111, and γTyr-117 (Chiara & Cohen, 1997; Chiara *et al.*, 1998) have all been labeled and thus play a role in ligand binding. The labeled amino acids from the γ/δ subunits may be responsible for the selectivity of ligands between the two non-equivalent binding sites such as d-tubocurarine which displays two distinct affinities for the displacement of ACh (Neubig & Cohen, 1979). When subunits were expressed in triplets consisting of αβγ and αβδ, two different binding affinities were found for d-methyltubocurarine and lophotoxin (Sine & Claudio, 1991). Although the two binding sites are closely related, contributions by the homologous γ/δ subunits may result in the non-equivalency of the two binding site (Sine, 2002).

The multiple binding loop hypothesized by Corringer *et al* (1995, 2000) postulates that the α-subunits constitute the principle binding compononent and the non-α subunits associated with the α-subunits provide a complementary binding component. Loops A-C were proposed to exist on the α-subunits and Loops D-F were contributed by the associated γ/δ subunits. The amino acids associated with these binding loops are those that were labeled previously using photoffinity labeling. Loop A consists of the αTyr-93 residue, αTrp-149 forms loop B, and αTyr-190, αCys-192, and αCys-192 form loop C on the α-subunits. The complimentary component is formed by γTrp-55, γGlu-57 and δTrp-57 of loop D, γLys-109, γLys-111 and δArg-113 from loop E, and γAsp-174 and δAsp-180 from loop F. The ligand binding pocket is thus formed as the polypeptide chains of each subunit's contributory amino acids come into close contact and stabilize the ligand within the binding site.

Evidence for multiple ligand binding sites of the nAChR

From the previously presented evidence, it was clear that there existed two distinct agonist binding sites located at the interfaces of the α/γ and α/δ subunits. A discrepancy exists between the agonist concentration required to activate the receptor (10-100 μM) and that measured in equilibrium binding assays (K_d~10 nM) (Raftery *et al.*, 1983). Explanations for the vast differences in measured ACh concentrations led to the speculation that the measured high affinity (K_d~10 nM) binding reflected the desensitized state of the receptor, which it was hypothesized had a much higher affinity for agonist than the open state (Quast *et al.*, 1978; Raftery *et al.*, 1983). The processes of activation and desensitization were envisioned to be a linear process mediated by the two high affinity binding sites. Alternatively, it was proposed that there may exist a number of distinct binding sites within the receptor which may be responsible for activation and desensitization.

Evidence for distinct binding sites present on the nAChR has been obtained using fluorescence affinity labeling techniques (Dunn *et al.*, 1980). The fluorescent probe 5-iodoacetamidosalicylic acid (IAS) was targeted to a reduced disulphide bond between Cys-192 and Cys-193 close to the high affinity binding sites. Binding of carbamylcholine to IAS-labeled nicotinic receptors caused a saturable enhancement of fluorescence which could be inhibited by either α-bungarotoxin or bromoacetylcholine (BrACh). These findings suggested that the sites labeled by IAS were near to agonist binding sites and that a change in fluorescence could be correlated to ligand binding. Furthermore, the measured dissociation constant in this study was comparable to others indicating that IAS most likely reflected high affinity binding.

Use of another fluorescent probe, 4-([(iodoacetoxy)ethyl]methylamino-7-nitro-2,1,3-benzoxadiazole (IANBD), revealed that binding of either ACh of CCh enhanced fluorescence but at much higher concentrations (10 μM-10 mM) than that required to increase fluorescence in IAS-labelled receptors (Dunn & Raftery, 1982a; Dunn & Raftery, 1982b; Dunn *et al.*, 1983). The much lower affinity revealed by fluorescence changes resulted in it being termed the low affinity binding site(s). The fluorescence enhancement could be abolished with the pre-incubation of the preparation with α-bungarotoxin, but was not susceptible to inhibition by other antagonists or covalently bound bromoacetylcholine. As IANBD fluorescence was not susceptible to these antagonists as had previously been found using IAS, it was proposed that IANBD monitored a site distinct from the classical high affinity binding sites. The dissociation constants measured were approximately 1000-fold higher than those measured from IAS studies and these higher dissociation constants were close to the concentrations required to activate the receptor in functional studies. These studies suggested that two classes of binding sites differentiated by their affinity for agonists as well as their susceptibility, or lack thereof, to various antagonists and that these two classes of sites may play different roles in the activation and desensitization of the receptor.

Further evidence for the existence of the two distinct classes of binding sites was uncovered by labeling nAChR simultaneously with both IAS and IANBD (Dunn & Raftery, 1993). From their data, the authors found that IAS and IANBD did monitor the binding of agonist at the same site. This study confirmed many of the previously observed properties of the two distinct sets of binding sites. The IAS fluorophore exhibited a saturable enhancement in fluorescence at much lower concentrations than the IANBD fluorophore. Furthermore, the IAS fluorophore was susceptible to agonist-induced fluorescence changes whereas IANBD was not. Since the two fluorescent probes were incorporated into the same receptor and still exhibited the properties previously found individually, this provides strong evidence for the hypothesis that these two labeled sites are indeed independent from another and may play a functional role in the activation and desensitization of the receptor.

Thus far, it has been suggested that the nAChR contains two high affinity binding sites located at the interfaces of the α/γ and α/δ subunits and may also potentially contain two low affinity binding sites that are distinct from the former sites. Thus, the nAChR potentially contains at least three or more agonist binding sites that regulate receptor activation and desensitization. Study of the kinetics of association and dissociation using radiolabeled ligands and fluorescence techniques has suggested that the two high affinity sites may consist of two subsites that are mutually exclusive at equilibrium (Dunn & Raftery, 1997a; Dunn & Raftery, 1997b). In one set of experiments, the addition of unlabeled agonist into a system in which tritiated ACh had equilibrated and occupied all high affinity binding sites resulted in an increase in its dissociation rate. At equilibrium, it was hypothesized that ACh occupied subsite A in the high affinity binding pocket. If a higher concentration of unlabeled agonist was added which subsequently bound to subsite B, it was found that the affinity of ACh for site A was reduced resulting in its dissociation. The bisfunctional ligand [^{3}H]suberyldicholine, under equilibrium binding conditions, was only slightly affected by the addition of unlabeled agonist (either ACh or SubCh) which suggested that SubCh could bridge the two subsites simultaneously and either bind or prevent the binding of other agonists. Examination of the kinetics of binding of CCh and SubCh using stopped-flow techniques and IAS fluorescence revealed that these two agonists had different association kinetics. Dunn and Raftery (1997b) found that the association kinetics for CCh occurred in three distinct phases, whereas the association kinetics of SubCh occurred in only two phases. This extra phase could only be interpreted as a second ligand binding step. The biphasic nature of SubCh association kinetics suggested that the bisfunctional nature of SubCh allowed it to span the two subsites and thus prevent another agonist molecule from occupying subsite B, demonstrated by the lack of a third association phase seen with CCh at higher concentrations.

From the evidence presented, it is clear that ligand binding is a much more complex process than previously considered. The classical concept involving two high affinity binding sites is unable to account for these new findings and therefore a more thorough kinetic model of the activation of the receptor is required.

CHANNEL GATING

The coupling of agonist binding to the receptor and the removal of the barrier to ion flow within the channel is a complicated process involving the rearrangement of a number of residues within a very short period of time. As described in the previous section on the three-dimensional structure of the nAChR, Unwin reports that the rotation of the inner β-sheets of the binding pocket push the α-subunits toward their associated subunits. The movement of the Cys-loop may result in its contact with the M2-M3 linker, the movement of which may be transmitted down the M2 helices, thus removing the kink near the gate and allowing current to flow. In general concurrence with the Unwin mechanism, modeling of agonist binding to the acetylcholine binding protein suggests that occupancy of the binding pocket initiates a conformation change in the Cys-loop, tilting the loop inward and bringing the conserved residues αTyr-180 and αLys-145 closer together, displacing αAsp-200 and weakening its interaction with αLys-145 (Celie *et al.*, 2004; Gao *et al.*, 2005). These inter-residue displacements may be translated to the channel through the β-strand which forms part of the Cys-loop and thus propagated down through the protein to the gate.

Other evidence suggests that the amino acid proline 272 in the M2-M3 linker plays a large role in channel gating (Lummis *et al.*, 2005). The side chain of the proline residue forms a closed loop which allows it to exist in either the *cis* or *trans* conformations. Lummis *et al.* (2005) found that unnatural amino acid substitutions that preferred the *trans* conformation impaired or eliminated activation of the receptor by agonist, whereas amino acid substitutions that preferred the *cis* conformation facilitated channel gating. Further complexity is revealed in the example of the amino acids Arg 209 and Glu-45 which have been shown to also alter channel gating (Lee & Sine, 2005). If the charge is reversed on either residue, channel gating is either impaired or abolished. On the other hand, if the charges are reversed in either position for both residues, gating is restored to levels approaching the wild-type receptor. A similar effect was demonstrated by Sala *et al.* (2005) in the α_7 homomeric nAChR. Charged amino acids located on loops 2 and 7 were found to be essential to gating function. If the charges at these positions were removed, virtually all whole-cell current was abolished. Furthermore, the creation of a double reverse charge mutant did not restore channel gating as might have been predicted if gating involved a simple electrostatic interaction among amino acids. It is clear that the mechanism of channel gating remains a mystery, however it is becoming clear that it involves the complex interplay of numerous amino acids to translate agonist binding to the channel gate many Ångstrom units distant.

DEVELOPMENT OF THE SINGLE CHANNEL PATCH CLAMP TECHNIQUE

Before the advent of the single channel patch clamp technique and its ability to observe, in real time, the opening of a single ion channel, the conductance and kinetics of ion channels had to be inferred from both whole cell recordings and noise analysis. By pressing a small diameter (3-5 µm) glass electrode onto a denervated frog muscle fibre, Neher and Sakmann (1976) were the first to report recordings of single channel currents. Their findings confirmed many of the previous hypotheses of the nature of ion channel activation, perhaps the most important of which was that the channel opened and closed almost instantaneously, producing a square current pulse on their recordings as was hypothesized by Katz and Miledi (1972). Furthermore, Neher and Sakmann found that the conductance of the nAChR measured via single channel analysis was similar to that estimated by noise analysis (Colquhoun *et al.*, 1975); the measured voltage sensitivity was within the range previously determined by voltage clamp experiments (Anderson & Stevens, 1973), and an agonist-dependent open channel duration was observed with single channel analysis as was reported using noise analysis (Katz & Miledi, 1973; Colquhoun *et al.*, 1975). The development of the single channel patch clamp by Neher and Sakmann was a giant leap forward in the field of electrophysiology, although a few technological hurdles needed to be overcome before the technique could be used to full effect. The rudimentary patch clamp amplifier and recording system did not possess the sampling frequency required to make accurate kinetic measurements of channel activity. Furthermore, as the patch seal was only 50 MΩ, there was a large amount of background noise which would create difficulties in discerning low conductance events. Finally, since the resistance of the seal was low, it precluded the use of techniques that could isolate the membrane from the cell which would be required for many types of experiments.

Most of these limitations were overcome with the introduction of improved patch clamp techniques (Hamill *et al.*, 1981). Instead of pressing the electrode against a clean cell or muscle fibre and then recoding from it, Hamill *et al.* increased the quality and strength of the seal by applying light suction to the interior of the patch electrode. This had the effect of increasing the resistance of the seal more than 100-fold thus creating a mechanically stable seal with excellent, low noise (a 10-fold reduction in baseline noise), characteristics. These giga-seals allowed the investigator to study single channel activation in new ways. Not only was the cell-attached method of recording more stable and of lower background noise than before, but the recording electrode could now be withdrawn from the cell to create cell-free patch recording in two different configurations. The inside-out patch configuration maintains the orientation of the membrane – the outside of the cell membrane is exposed to the inside of the patch pipette while the cytoplasmic face of the membrane is exposed to the bath solution. For the outside-out patch, the opposite is true. These two configurations allow the study of ion channels at a much higher resolution than was possible before and have contributed greatly to the knowledge of the functional characterization of receptor activation.

Further improvements to the single channel patch clamp have focused on reducing the background noise of recordings. Low-noise amplifiers based on those first developed by Neher and Sakmann (1976) and Hamill *et al.* (1981) have been developed which attempt to remove all sources of extraneous electric, thermal, and vibrational sources of background noise all in an effort to increase the signal to noise ratio. This has been the most popular method used by single channel electrophysiologists to increase the quality of recordings. Another method, less popular due to expense, is the use of quartz electrodes pulled using a laser. These electrodes possess electric properties that significantly reduce background noise, especially high frequency noise, thus allowing recordings to be made at much higher filter frequencies (Rae & Levis, 1992; Levis & Rae, 1993; Parzefall *et al.*, 1998). The newest single channel patch clamp technique, the optical patch clamp, is gaining popularity among some groups, although the technology is still in its infancy. The technique uses calcium fluorescent dyes and a highly sensitive camera to detect fluorescence transients emitted as channels open and allow calcium to enter the cell (Borisenko *et al.*, 2003; Demuro & Parker, 2005). Optical patch clamp allows the recording of numerous channels simultaneously, thus allowing a large sample size to be accrued rapidly. One major drawback of this technique is the time resolution of the calcium-sensitive dye. At 2 ms, it is not quick enough to capture many of the rapid channel openings/closures to adequately characterize the kinetics of the channel being studied. Furthermore, the technique requires the channel under investigation to be permeable to Ca^{2+} which limits its usefulness to other ion channels that are Ca^{2+}-

impermeable. Whatever the future of the technology of the single channel patch clamp, the technique will remain integral to the functional characterization of ion channels.

THE SUBCONDUCTANCE STATE

Early models of channel activation included but two states: open and closed. Katz and Miledi (1972) proposed that the opening and closing of the receptor was a sudden on/off event that allowed a unitary current to flow and the summated duration of all the unitary current accounted for the exponential decline of the end plate current. With the introduction of single channel recording by Neher and Sakmann (1976) and its refinement by Hamill *et al.* (1981), individual channel openings could be observed without the need for Fourier transforms. The predictions made by Katz and Miledi were proved largely to be true. However the on/off theory of channel activation proved to be incomplete. It was soon observed that a single channel of the nAChR could open to multiple conductance states, usually two (Hamill & Sakmann, 1981; Brehm *et al.*, 1982; Trautmann, 1982; Auerbach & Sachs, 1983; Auerbach & Sachs, 1984; Takeda & Trautmann, 1984; Morris & Montpetit, 1986; Morris *et al.*, 1989). These states are broken down into two categories: the main, or full conductance open state, that generally comprises the majority of openings and which is considered the largest conductance of the ion channel and the subconductance state(s) which have smaller single channel conductances than the main state. Further study has revealed that other ligand gated ion channels exhibit subconductance openings including GABA$_A$ receptors (Hamill *et al.*, 1983; Bormann & Clapham, 1985), glutamate receptors (Cull-Candy & Usowicz, 1987; Cull-Candy & Usowicz, 1989; Usowicz *et al.*, 1989; Cheffings & Colquhoun, 2000), glycine receptors (Hamill *et al.*, 1983; Bormann *et al.*, 1993), 5-HT$_3$ receptors (Davies *et* *al.*, 1999), and the P2X receptor (Whitlock *et al.*, 2001). LGICs are not the only channels where subconductances have been reported. Multiple conductance states have been observed in potassium channels (Labarca & Miller, 1981; Tomlins *et al.*, 1984), chloride channels (Tank *et al.*, 1982; Hanke & Miller, 1983), and gramicidin channels (Busath & Szabo, 1981; Woolley & Wallace, 1992).

Criteria for recognition of the subconductance state

In his review of the subconductance state, Fox (1987) laid out a set of criteria to be used in it recognition. Firstly, the subconductance state should interconvert with the channel main state and these interconversions should be readily observed. The second criterion for the recognition of the subconductance state is that it should be observed in conjunction with the main, or full conductance state. If the subconductance state is observed exclusively within a recording, the most likely explanation is that it is a separate channel, not a substate of the channel that produced the main open state. Finally, great care must be taken to correctly identify whether the main state is the superimposition of two independent channels or is, in fact, a single channel with multiple conductance states. Although not as definitive as the previous criteria, examination of the ionic selectivity of both the main and substates may provide further evidence that the observed subconductance current is, at least, the same type of channel that produces the main conductance. Caution must be taken, however, as Fox points out that some receptors display different ionic selectivities among conductance states (Busath & Szabo, 1981). Using these criteria, it should be possible to distinguish between a true subconductance state and a patch containing multiple channels and therefore eliminate a possible source of error in the interpretation of single channel currents.

Hypotheses for the existence of the subconductance state

A number of hypotheses have been proposed to explain the subconductance state: one postulates that the subconductances arise from the partial block of the channel by an exogenous agent, the other proposes that subconductance currents are the result of multiple conformations adopted by the receptor. An earlier hypothesis, proposed by Sakmann *et al.* (1978), had suggested that subconductances are an artifact of the recording system in that they are the result of rim channels. The partial occlusion of the channel pore by the rim of the recording electrode produces a current that is lower than would otherwise be recorded if it were not blocked therefore producing what appears to be a distinct subconductance state. Improved patch clamp recordings developed by Hamill *et al.* (1981) allowed for a much smaller area under the rim of the pipette which drastically decreased the frequency of rim currents to the point of becoming inconsequential.

The partial block hypothesis was first proposed by Takeda and Trautmann (1984) from their study of the actions of d-tubocurarine (curare) on rat myotubes. It suggests that the subconductance state is the result of a fully open channel which is partially occluded by a charged molecule such as an agonist which, when bound to a site within the channel vestibule, decreases the flow of ions through the gate. Although ions pass through the gate in single file and therefore any vestibular occlusion would require it to be almost total in cross-sectional area to affect this, the hypothesis took into account the alterations to the charge distribution within the vestibule introduced by the binding of a charged molecule. The interruption in the lines of force around the receptor thus caused could be envisaged to affect ion transition.

Though classically an antagonist, curare was found to be a weak agonist of the nAChR in some systems (Ziskind & Dennis, 1978; Jackson *et al.*, 1982; Trautmann, 1982; Morris *et al.*, 1983; Morris *et al.*, 1989). Takeda and Trautman found that curare elicited currents that had an amplitude 40% that of the full conductance, or main current, and that these currents were slightly curare concentration-dependent. Furthermore, the authors found that the frequency of subconductance currents could be increased by hyperpolarizing the patch. This model was only slightly favoured in their analysis as another model, one in which the channel opens partially, also fitted the available data. The authors proposed that the partial block binding site must be located in a position more external to the gate than that of the classical full channel block site. It was found that the partial block site could be occupied while the channel was in the closed conformation as the channel could then open directly to the subconductance state. This is in contrast to the full channel block site which requires the channel to be in the open conformation before the blocking site can be occupied by a ligand to interrupt the ionic current completely (Colquhoun *et al.*, 1979). From their data, Takeda and Trautmann implied that there must exist binding sites other than the two agonist binding sites associated with the α-subunit and that these distinct sites were most likely located within the channel vestibule.

Further evidence for the partial block hypothesis was produced by Strecker and Jackson (1989). These authors found that even if the two classical agonist binding sites were purportedly made agonist-insensitive by successive reduction and alkylation with dithiothreitol and N-ethyl maleimide, curare-induced subconductance openings remained relatively constant in frequency. Since the binding sites had been inactivated, curare, or any agonist, would have been unable to activate the receptor conventionally; most openings in this system ought to be spontaneous. Strecker and Jackson argued that this was evidence for the partial block hypothesis where curare was acting to attenuate the amplitude of the spontaneous currents. They did, however, concede that they could not rule out the possibility that other distinct binding sites not within the channel ion path could produce similar results without causing outright channel block.

In their paper describing the multiple conductance states of the nAChR, Hamill and Sakmann (1981) proposed a mechanism whereby a single channel could exhibit more than one level of conductance. They proposed that the conformational flexibility of the five homologous subunits could result in the channel producing multiple conductance states. The subconductance current, elicited by ACh, was approximately 40% that of the main state and opened relatively infrequently. Subconductance openings observed by Auerbach and Sachs (1983, 1984) were qualitatively different from those reported by Hamill and Sakmann (1981) in that they were extremely rapid flickers to the substate from the main conductance state. Most of the subconductance openings lasted less than 250 μs and had an amplitude approximately 12% that of the main state. The authors ruled out channel block as an explanation of the subconductance state as the concentrations of ACh used were 50-250 nM and suggested that the rapid flickering to the substate may represent closures of the doubly liganded state.

Perhaps the most extensive study of the subconductance state was performed by Morris and Montpetit (1986) who also subscribed to the multiple conformations hypothesis of subconductance openings. The authors examined the ability of a number of nicotinic agonists including ACh, choline, carbachol, nicotine, succinyldicholine, suberyldicholine, and curare to elicit subconductance currents. All of the above agonists were able to activate the receptor and produce the main conductance state as well as elicit subconductance currents, the amplitudes of which were relatively constant among the different compounds. Some agonists (ACh, Carb, SubCh, curare) produced two distinct subconductance amplitudes which were 12% and 8% that of the main state, while the remaining agonists only elicited the lower subconductance state. The authors concluded that the structures of the various agonists may impart a higher affinity for one or both subconductance states thereby increasing the probability of opening to these substates. In the partial channel block model, it would be expected that larger molecules would produce subconductance openings with a lower channel conductance as they would occlude a greater portion of the channel and therefore allow less current to pass through the pore. The data suggested, however, that there was no relation between the molecular weight of the compound and the appearance of subconductance currents, thus presenting a strong case against the partial block hypothesis. Morris and Montpetit (1986) also examined spontaneous channel openings in the absence of any nicotinic agonist in search of subconductance currents. They reported that of the infrequent events captured, a few appeared to be subconductance openings; however the small sample size precluded further study. If they had been able to unequivocally show spontaneous subconductance openings, that would have been a strong argument against the partial block. The authors concluded that binding of agonist to the receptor increases the likelihood that the channel will enter not just the main open state, but also increases the likelihood that the channel can enter a group of open states, the kinetics of which are dictated by the properties of the agonist used.

Theoretical modeling of the subconductance state

Theoretical modeling by Dani and Fox (1991) has attempted to explain how the channel can adopt multiple conformations that would be manifested as multiple conductance states. Three models were proposed to explain how a single pore ion channel can produce regular subconductances and main conductance that possess the same selectivity and ion binding affinity. The first model simulated an ion channel that could exist in a number of long-live conformational states, each of which would have different energy profiles for ion permeation. The selectivity of the channel need not be altered by changes in one or more energy barriers to permeation as the offset can be compensated for by other barriers. As it is difficult to produce detectable changes in conductance with this model, the authors concluded that separate open states may only be distinguishable under particular experimental conditions. This model is useful, however, in providing evidence for a mechanism whereby the receptor can adopt multiple conformations without losing ion selectivity.

The second model proposed by Dani and Fox (1991) considered a channel that rapidly fluctuated between two conformational states which had different energy profiles for ion permeation. Since the conformational changes in this model are much more rapid than the open duration of the channel under normal conditions, the frequency of lower conductance openings of the channel result from changes in the vibrational transitions of the channel between two stable conformations, one of which has a lower energy barrier to the permeation of ions, the other higher. Since the subconductance state is not in itself a stable conformation of the receptor, any exogenous agent that perturbs the system at equilibrium will result in a change in the frequency of subconductance openings. For example, if an agonist upon binding to the receptor slows down the conformational change between the two states, the ions permeating through the channel are more likely to encounter a higher energy barrier to their movement and therefore a subconductance current would result.

The third and final model proposed by Dani and Fox hypothesized that superficial changes to the vestibule of the channel may produce subconductances. If the charge distribution of the vestibule were to be altered so that only a fraction of the negative charge could act on the permeating cations, a slower flow of these ions would result and subconductance openings would occur. This model does not require changes in the conformational states of the receptor that produce the closed and full conductance open states, but rather only minor perturbations of these conformations that would alter the net negative charge of the vestibule.

All three models provide a plausible mechanism for the appearance of subconductance openings, however experimentation to determine which of the three is correct is very difficult. Since many of the conformational changes are extremely rapid and the currents involved are very small, electrophysiological recording methods (ie. single channel patch-clamp recording) are not possible. In fact, since the publication of this paper, no other group has attempted to experimentally test these hypotheses. These models, although untested, provide a framework for discussing the underlying mechanism of the phenomenon of subconductances.

AIMS OF THE PRESENT STUDY

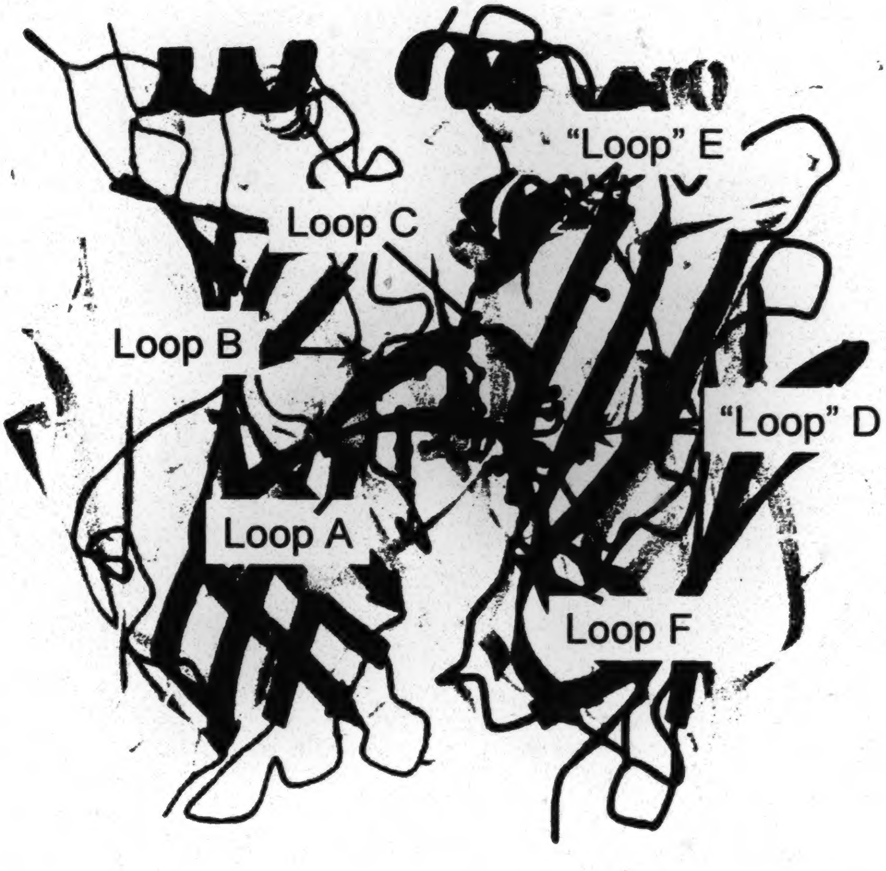
The occurrence of subconductance currents has long been acknowledged, however the mechanism by which the channel opens to a lesser conductance state is little understood (Hamill & Sakmann, 1981; Auerbach & Sachs, 1983; Auerbach & Sachs, 1984; Morris & Montpetit, 1986; Dani & Fox, 1991; Morris *et al.*, 1989). One theory proposes that subconductance currents arise from the partial occlusion of the channel pore by an exogenous agent (Takeda & Trautmann, 1984; Strecker & Jackson, 1989) while an alternate hypothesis posits that subconductance currents arise from the stabilization of a distinct conformation state of the receptor which possesses a lower permeability to ions (Hamill & Sakmann, 1981; Morris & Montpetit, 1986).

There are two classic high affinity binding sites contained on the nAChR, the occupancy of which activates the receptor. With only two binding sites, it becomes difficult to account for subconductance currents if the two high affinity sites are responsible for the duration of full conductance currents (Labarca *et al.*, 1984a; Labarca *et al.*, 1984b; Labarca *et al.*, 1985). Evidence from equilibrium and kinetic studies has revealed that there may be, in fact, other sites located within the receptor that are capable of ligand binding and indeed are responsible for receptor activation (Dunn *et al.*, 1980; Dunn *et al.*, 1983; Dunn & Raftery, 1982a; Dunn & Raftery, 1982b; Dunn *et al.*, 1993; Dunn & Raftery, 1997a; Dunn & Raftery, 1997b).

This study will examine the activated states of the nicotinic acetylcholine receptor as a function of agonist type and concentration. By examining the frequency of subconductance currents evoked by a number of nicotinic agonists over a large concentration range, a relationship between receptor occupancy and the generation of subconductance currents may be determined. Furthermore, if the distinct binding sites possess different affinities for ligands, it will be possible to separate the sites responsible for the subconductance and full conductance states. The semi-rigid agonists, DMPP and PTMA will be investigated as it has previously been reported that an agonist of similar structure, methyl arecolone, elicits a large fraction of subconductance currents (Kawai *et al.*, 2000),. These compounds, by virtue of their semi-rigid structure, were hypothesized to inhibit conformational changes within the receptor necessary for a full conductance opening. We will examine whether this is true for all semi-rigid agonists and for all concentrations. Finally, we will determine which of the two models proposed to describe the underlying mechanism of subconductance generation is most probable.

Figure 1-1

Ribbon diagram of the acetylcholine binding protein (AChBP) pentamer viewed perpendicularly to the five-fold axis. This is homologous to the N-terminal region of the nicotinic acetylcholine receptor (nAChR). Two of the five protomers are highlighted in colour. Three binding loops (Loops A-C) form the principle binding component of the α-subunit of the nAChR. These loops are located on the yellow protomer on the AChBP diagram and are labeled in black. The complementary binding loops (D-F) are located on the γ- and δ-subunits of the junctional nAChR. In this diagram, they are located in the blue protomer and are labeled in red. Figure modified from Brejc *et al.* (2001).


"Loop" E
Loop C
Loop B
"Loop" D
Loop A
Loop F

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CHAPTER 2

Activation of the nicotinic acetylcholine receptor by acetylcholine

Introduction

In their detailed paper introducing noise analysis as a technique for the investigation of nicotinic acetylcholine receptor (nAChR) function, Katz & Miledi (1972) speculated that the current pulse following channel activation was a quantal event, incapable of subdivision, and with a virtually instant onset and offset profile. The initial direct measurement of single channel currents (Neher & Sakmann, 1976) seemed to substantiate this prediction, but with the refinement of the patch clamp technique (Hamill *et al.*, 1981), it became obvious that a single channel could exhibit more than one conductance (Hamill & Sakmann, 1981; Auerbach & Sachs, 1983, 1984). This observation has subsequently been extended to other ligand gated ion channels of the same and other superfamilies, and even to voltage gated channels (Hamill *et al.*, 1983; Bormann & Clapham, 1985; Fox, 1987; Angelotti & Macdonald, 1993; Bormann *et al.*, 1993; Caitlin Smith *et al.*, 2000; Cheffings & Colquhoun, 2000; Whitlock *et al.*, 2001; Premkumar *et al.*, 2002).

Although the case that the lesser or sub-conductance current was a true manifestation of a channel state, and not an artefact of the measuring system, was eloquently made by Sachs (1983) and reviewed by Fox (1987); kinetic analyses have subsequently focussed on the bimodal nature of the full conductance channel open times on the premise that this might be correlated with the single or double occupancy of two binding sites presumed to exist in the receptor oligomer (Labarca *et al.*, 1984, 1985; Jackson, 1988). As a result, the subconductance state has languished as an object of investigation, being dismissed as occurring too infrequently to be physiologically significant (Edmonds *et al.*, 1995), or an inconvenience that was digitally eliminated from records to facilitate computer analysis (Elenes & Auerbach, 2002). Nonetheless, subconductance currents of nicotinic receptors have been associated with a variety of other ligands, frequently with a rigid or semi rigid component in their structure (Trautmann, 1983; Takeda & Trautmann, 1984; Morris & Montpetit, 1985; Morris *et al.*, 1988; Strecker & Jackson, 1989). In 2000 we reported that at 5 μM carbachol (CCh) both full and subconductance currents could be discerned on recordings, yet at 2 μM methylarecolone only subconductance currents were visible despite the fact that this agonist was three-fold more potent than acetylcholine (Kawai *et al.*, 2000). This suggested that the subconductance state was of physiological significance and represented a distinct activated state worthy of further investigation.

The results indicate that the proportion of subconductance currents evoked by acetylcholine (ACh) or CCh bears an inverse relationship to the concentration of agonist, and that these currents form part of a homogeneous population that should be included in kinetic models of receptor activation.

Materials and Methods

Preparation of nAChR-enriched membrane fragments

Membrane fragments, highly enriched in nAChRs, were prepared as described by Elliot *et al.* (1980) using frozen *Torpedo californica* electroplax from Aquatic Research Consultants (San Pedro, CA). To prevent enzymatic degradation of receptors, preparation of membrane fragments was performed at 4°C and in the presence of the enzyme inhibitors EDTA, phenylmethylsulphonylfluoride (PMSF, Sigma-Aldrich Canada, Oakville, ON), and pepstatin A (Fluka BioChemika, Buchs, Switzerland). *Torpedo* electric organ (0.5 kg) was removed from storage (-86°C) and broken into small fragments with a hammer. These fragments were suspended in 500 mL of homogenization buffer (10 mM sodium phosphate, 0.4 M NaCl, 5 mM EDTA, 1 mM phenylmethylsulphonylfluoride, 5 mM iodoacetamide, 0.05 mg/mL pepstatin A, 0.02% NaN_3, pH 7.8) and homogenized on the high setting of a Waring blender for two 1 minute periods with a one minute rest in between.

Aliquots of the membranes were re-homogenized using a Virtis 45 (Gardiner, NY) for four 30 second periods each interspersed with a 30s period of rest at a setting of "50". The aliquots were centrifuged in a Sorvall RC-5B centrifuge in a GSA rotor (Thermo Electron Corporation, Waltham, MA) at 5000 rpm for 10 minutes and a temperature of 4°C. The supernatant was poured through four layers of cheesecloth securely fastened over the top of the centrifuge containers. The homogenate was then centrifuged at 30000 rpm for 1 hour at 4°C in a 45 Ti rotor using a Beckman L-70 Ultracentrifuge (Beckman Coulter Ltd, Fullerton, CA). The pellets were resuspended in approximately 40 mL of low salt buffer solution (10 mM sodium phosphate, 1 mM EDTA, 0.02% NaN_3, pH 7.4) and homogenized for two 30 second periods using a Virtis 45 homogenizer at a setting of "50".

The resuspended material was weighed and sucrose and NaCl was added to give a final concentration of 30% (w/w) sucrose and 0.4 M (2.3% w/w) NaCl. This solution was stirred for approximately 20 minutes at 4°C to allow the sucrose to fully dissolve. A discontinuous sucrose gradient was prepared using the following volumes and concentrations (% w/w sucrose in Buffer B, see below) in a Beckman VTi 50 centrifuge tube: 5 mL 50%, 5mL 39%, 12 mL 35%, 15 mL homogenate (30% w/w sucrose), and 1 mL overlay of Buffer B (10 mM sodium phosphate, 0.4 M NaCl, 1 mM EDTA, 0.02%

NaN_3, pH 7.4). The VTi 50 rotor was placed in the L-70 ultracentrifuge and centrifuged for 60 min at 45000 rpm and 4°C. A slow acceleration setting was used until the centrifuge reached 2000 rpm and the brake was turned off before the centrifuge slowed to less than 2000 rpm so as not to disturb the sucrose gradient. The middle band was collected, diluted three-fold with ice cold Buffer B and centrifuged in a 45 Ti rotor in the L-70 Ultracentrifuge for 60 min at 30000 rpm and 4°C. The pellet was resuspended in 20 mL of resuspension buffer (10 mM Hepes, 100 mM NaCl, pH 7.4) using the Virtis 45 homogenizer at a setting of "50".

To determine the concentration of total protein of the homogenate, a Lowry protein determination assay (Lowry *et al.*, 1951) was performed. Immediately before the assay was started, 0.5 mL of Solution B (2.3% w/v Na K tartrate) was added to 0.5 mL of Solution A (1% w/v $CuSO_4 \bullet 5H_2O$) and made up to 50 mL with Solution C (3% w/v Na_2CO_3, 0.4% w/v NaOH) to create the Lowry reagent. A stock solution containing 1 mg/mL bovine serum albumin (BSA; Sigma-Aldrich Canada, Oakville, ON) was used as the protein standard. A series of tubes was prepared with BSA concentrations of 0 mg/mL, 1 mg/mL, 2 mg/mL, 5 mg/mL, 10 mg/mL, 15 mg/mL, 20 mg/mL, and 25 mg/mL and subsequently diluted 1:100 and 100 μL of each concentration was used. The membrane sample was diluted 1:20 in distilled water and a series of concentrations was prepared to mirror the volumes added to create the BSA protein standard curve. These samples were mixed with 1mL Lowry reagent and allowed to incubate at room temperature for 30 minutes. After the incubation period, 100 μL of Folin's Reagent 1N (1:1 dilution with distilled water; Sigma-Aldrich Canada, Oakville, ON) was added, vortexed immediately, and allowed to incubate at room temperature for 15 minutes. The absorbance was read using a Beckman DU-40 spectrophotometer (Beckman Coulter Canada Ltd, Mississauga, ON) at a wavelength of 650 nm. The results were plotted and the concentration of undiluted nAChR-enriched membranes was determined and diluted to a final concentration of 1 mg/mL. The membrane preparation was aliquoted into 150 μL samples and stored at -86°C until required.

Preparation of giant liposomes

Giant liposomes were created via the dehydration/rehydration technique as described by Riquelme *et al.* (1990). L-α-phophatidylcholine (Sigma-Aldrich Canada, Oakville, ON) was suspended in 10 mL of distilled H_20 via sonication (W-375 Sonicator, Heat Systems-Ultrasonics Inc., Plainview, NY) for ~2-3 minutes. The suspension was then diluted 1:10 with 10 mM HEPES, 100mM NaCl, pH 7.4 and solid CHAPS (Fisher Scientific Canada, Edmonton, AB) was added to final concentration of 1% (w/v). Formation of lipid vesicles was accomplished via dialysis over a period of 4-5 days at 4°C. These dialyzed vesicles were divided into 2 mL aliquots and stored at -86°C until required.

To reconstitute the receptors into giant liposomes, 150 μL of thawed membrane fragments were added to ~2 mL of thawed, dialyzed vesicles and centrifuged (4°C) for 1hr at 100000 g in a Beckman 45Ti rotor using a L-70 Ultracentrifuge. The supernatant was discarded and the pellet resuspended in 0.1 mL of 50 mM NaCl, 4 mM HEPES, pH 7.4 and 5% (v/v) ethylene glycol. The suspension was homogenized by passing it through a 26 gauge needle numerous times. Small drops (~20 μL) were deposited onto 35 mm plastic dishes and placed in a dessiccator containing anhydrous $CaCl_2$ and allowed to dehydrate overnight at 4°C. Rehydration involved adding ~20 μL of experimental buffer (50 mM NaCl, 4 mM HEPES, pH 7.4) onto each of the dehydrated drops and incubated for 2-3 hours at 4°C. The rehydrated drops were pipetted off the dish using experimental buffer and centrifuged for 15 minutes in Silencer H-25F1 bench centrifuge at 1000 g. The pellet was resuspended in 1 mL of experimental buffer and 3-4 drops were pipetted into a 35 mm polystyrene Petri dish (Nalge Nunc International, Rochester, NY) for experimental use.

Patch-clamp recording

Single channel recordings were performed in the inside-out patch configuration (Hamill *et al.*, 1981). Glass microelectrodes (Corning #0010, 1.5 mm OD, 0.75 mm ID, WPI, Sarasota, FL) were pulled using a Flaming-Brown P-87 micropipette puller (Sutter Instrument Company, Novato, CA), fire-polished with a DMF 1000 Microforge (WPI, Sarasota, FL) and coated with Sylgard #184 (Dow Corning, Midland, MI). Electrodes were filled with the recording solution containing 50 mM NaCl, 4 mM HEPES, 0.1 mM $CaCl_2$, and pH 7.4 which was ionically identical to the bath solution. Drugs were added to the pipette solution. Gigaseals were formed, often spontaneously, by placing the tip of the microelectrode (open resistance ~10 MΩ) against the surface of the liposome. All experiments were performed at 23°C and the electrode potential for all patches was set at +90 mV. Recordings were made using an Axopatch 200B Integrating Patch Amplifier (Axon Instruments, Union City, CA), connected to an external 8-pole Bessel filter (LBPF 48DG, npi Electronic, Tamm, Germany) set at a cutoff frequency of 2-3 kHz. Records were digitized via a Digidata 1322A (Axon Instruments, Union City, CA) and stored on a hard drive or CD-ROM using Strathclyde Electrophysiology Data Recorder software 2.4.9 (courtesy of Dr. J. Dempster), at a sampling frequency of 100 kHz.

Single channel analysis

Channel openings/closures were detected by using the threshold method included in the Strathclyde EDR software. To produce amplitude histograms, the threshold was set below the amplitude level of the subconductance state but above the level of the closed state. Amplitude histograms were fitted by a sum of Gaussian functions that used an iterative least squares fitting procedure. The height *y(i)*, of bin *i* with its mid-point at current *I(i)*, is given by

$$y(i) = \sum_{i=1..n} \frac{A_i w}{\sqrt{2\pi\sigma_i^2}} \exp\left(-\frac{(I(i)-\mu_i)^2}{2\sigma_i^2} \right)$$

where *w* is the histogram bin width. Each Gaussian component is defined by 3 parameters: A_i the percentage of the total area under the component, μ_i the mean current, and σ_i the standard deviation about the mean value. Software for this analysis was included in the Strathclyde WinEDR program. The results were displayed with Prism 4.0 (GraphPad Software, San Diego, CA).

For open duration analysis, the full and subconductance populations were analyzed separately. This was accomplished by setting the threshold above the sub-level but below the full level for analysis of the full conductance events. For analysis of the subconductance open durations, all events above the subconductance amplitude were ignored in the threshold event detection. Furthermore, for both full and subconductance currents, all events with a reported dwell time < 200 µs were excluded. Open durations and best fit population curves were plotted using the methods outlined by Sigworth & Sine (1987). Results are presented as mean ± s.e.mean unless otherwise stated. Statistical significance between open time constants was determined using an unpaired two-tailed t-test or one-way analysis of variance (ANOVA) where appropriate.

Results

Subconductance recognition.

Once a stable gigaseal was established, currents were clearly discernible as rapid changes in the recorded current level (Figure 2-1). At a sampling frequency of 0.1 MHz, and filtering at 2 KHz, the risk of aliasing was eliminated. This sampling rate ensured that a true representation of current change was captured over a 200 μs time period. With an 8-pole Bessel filter, the attenuation of an instant current change approximates $0.3/f_{-3}$ where f_{-3} is the filter cutoff frequency at -3dB. Therefore the filtered signal change reaches 90% maximum after 150 μs. A minimum dwell time of 200 μs could therefore be used as a criterion of a true subconductance and not merely a full conductance opening so rapid that the current profile is distorted by filtration and the full extent of the current transition is lost. Under those conditions subconductance currents were visible at all concentrations of both ACh and CCh examined. They were most obvious at low concentrations of agonist (< 1 μM) where the incidence of all currents was lowest (Figure 2-1a). At higher concentrations (Figure 2-1b & 2-1c) the subconductance currents were less obvious to the casual glance, but careful examination of the records revealed a population of subconductance currents interspersed between the larger full conductance events. The presence of the subconductance currents was further obscured by their shorter duration in comparison to that of the full conductance current (Figure 2-1d). At this concentration of agonist (1 mM), binding sites might reasonably be presumed to be saturated. The continued existence of subconductance currents speaks to autonomy in their generation.

Occasionally a subconductance current was combined with a full conductance current in time, but was never additive to the full conductance amplitude. Four clear types of subconductance could be identified. We have accorded them the following discriminators: Type 1 - A simple current that is unconnected to any other current and appears as an almost instantaneous change in current level from zero to about 1.7 pA before returning equally rapidly to zero (Figure 2-2a), Type 2 - A subconductance current that rises from baseline to a plateau maintained for at least 200 s, and then continues to a full conductance current value without returning to rest between (Figure 2-2b), Type 3 - A subconductance current that follows immediately after a full conductance current without an intervening excursion to the resting state (Figure 2-2c), and Type 4 - A subconductance current that is interposed between two full conductance currents, without return to rest between (Figure 2-2d).

Frequency of types.

The relative frequencies of the different types of subconductance in a series of recordings from different patches were viewed and the visible subconductance currents assigned to one of the four categories. Data from several patches at each concentration of ACh were combined and the pooled results are plotted in Figure 2-3. The type 1 subconductance currents showed overwhelming dominance at all concentrations tested (1-30 μM) and there was little obvious concentration dependent change in the distributions. Type 4 subconductances were slightly more frequent at 10 and 30 μM but the correlation is weak. The type 2 and 3 subconductances were rare, so much so that they could be safely categorized as random coincidences.

Amplitude.

Population plots of current amplitude at different concentrations of ACh reveal a clear separation into two populations with mean amplitudes of 2.7 pA (± 0.2 SD) and 1.75 pA (± 0.22 SD) which give chord conductances of 30.1 (± 0.8) and 19.1 (± 0.6) pS respectively (Figure 2-4a). These values were not affected by concentration. The corresponding values for carbachol are 2.77 pA (± 0.27) and 1.74 pA (± 0.18) (Figure 2-4b). These values compare well to values published for the reconstituted *Torpedo* receptor (Tank *et al*., 1983; Labarca *et al*., 1984). The clear separation of currents into distinct populations is a strong argument for the separate existence of the subconductance current as a distinct entity rather than assuming it to be the extreme of a continuous spectrum of current amplitudes.

Current duration distributions

The bimodal nature of the population of full conductance channel open times has long been recognized and used as a basis for kinetic modelling (Jackson & Lecar, 1982). Similar in-depth studies have not been reported for the subconductance currents. Accordingly we have analysed the channel open duration populations for both full conductance and subconductance currents at different concentrations of both acetylcholine and carbachol. A representative sample of the full conductance current distributions are shown as a Sigworth-Sine plot in Figure 2-5a where the bimodal nature of the current distributions is evident. The proportional contributions of the two populations of open channel durations as a function of ACh concentration are illustrated in Figure 2-5b, where a steady increase in the proportion of the longer duration currents is seen as a concomitant of rising concentration. The mean time constants for full conductance currents evoked by ACh are 0.52 (± 0.02) ms and 4.74 (± 0.05) ms and for CCh the equivalent values are 0.61 (± 0.08) ms and 2.7 (± 0.2) ms, respectively. These are in reasonable agreement with values published for *Torpedo* and other muscle type nicotinic receptors (Jackson *et al.*, 1983; Labarca *et al.*, 1985; Jaramillo & Schuetze, 1988). The subconductance currents, however, showed only one stochastic population with either agonist (Figure 2-5c), with a mean time constant of 0.43 (±0.01) ms for ACh and 0.49 (± 0.01) ms for CCh. The time constant values were not affected by concentrations up to 1 mM (Figure 2-6). At that point a significant reduction in mean open time occurred, which disappeared upon reversing the holding potential. The reduction in mean channel open time was therefore attributed to open channel block by ACh molecules.

Effect of concentration

The concentrations of either CCh (EC_{50}~1 mM, Moore & Raftery, 1980) or ACh (EC50~100 μM, Raftery *et al.*, 1983) used for most experiments reported here were considerably below the EC_{50} level as measured from rapid flux experiments. In this way, the risk of desensitization was minimized. The objective of our studies was to identify activated states of the receptor resulting from the partial occupation of the binding of the receptor. It became evident from our observations that the proportion of all events contributed by subconductance currents was sensitive to agonist concentration (Figure 2-7). At concentrations of ACh below 10 μM, subconductance currents were over 50% of all currents observed, and this proportion increased with lower concentrations. An identical relationship was obtained with CCh. The decline in the subconductance current fraction with concentration was not a manifestation of declining incidence (see Figure 2-1) but rather that the frequency of full conductance currents increased against an ongoing presence of subconductance currents.

Discussion

At other ligand gated ion channels, the presence of multiple conductance states is reported and accepted without comment, but at nicotinic acetylcholine receptors there has been controversy with a tendency to attribute the lower conductance either to a partial occlusion of the channel by the electrode rim (rim channels) (Neher *et al.*, 1978) or to separate channel types in the patch (Owens & Kullberg, 1989; Kullberg *et al.*, 1990). There has been reluctance to ascribe any importance to them due to either the limitations of conventional recording equipment (Colquhoun & Sakmann, 1985) or the infrequency of their occurrence (Edmonds *et al.*, 1995). Subconductance currents are, by definition, small in amplitude, and this may compromise their detection above background noise in some recordings. In addition, their average brevity also brings them close to the limit of resolution in standard filtering protocols. When data are examined with these limitations in mind, it is nevertheless possible to find unequivocal examples of currents where the signal to background noise ratio is greater than ten and where the dwell time at the peak of the current is sufficiently long as to reveal a clear plateau and not merely a spike of dubious amplitude. Such plateaux can be used to set the subconductance current amplitude, and it is noteworthy how often brief currents, that might otherwise be regarded as indeterminate spikes, attain the same current amplitude as the plateaux. This observation is reinforced by the frequency with which transient closures from full conductance current amplitudes characterized as types 3 or 4 subconductance currents drop to the same value to which the type 1, or 2 subconductance currents rise. In view of this constancy, it is unlikely that subconductances were merely incompletely captured full transitions from the closed to the open state and back. If that were the case, we might expect a range of amplitude values consonant with the range of open times and filtered by a constant capacitance.

Some authors prefer to regard the subconductance currents as rising from partial channel block caused by the agonist molecules themselves (Takeda & Trautmann, 1984; Green & Cottrell, 1999). In the present work, this seems an unlikely cause since subconductance currents predominate at concentrations of agonist in the nanomolar range where the chance of an agonist molecule being in the receptor vestibule is less than 1:100. The opportunity, then, of ACh molecules simultaneously occupying binding sites and entering the ion pore and blocking it is remote indeed. In addition, where unequivocal channel block occurs in the nAChR, it has a recognizable appearance of rapid flickering of the channel from the fully open to the closed state, and measurements of channel open time show a significant reduction (Auerbach & Sachs, 1983; Ogden & Colquhoun, 1985; Neher, 1983; Sine & Steinbach, 1984; Sine *et al.*, 1990). In our records, full conductance currents of normal open time coexist with type 1 subconductance currents over a wide range of agonist concentrations, and there is no evidence of significant amounts of channel flicker. As most of our subconductance currents arise from baseline (type 1), they cannot be interpreted as a reduction in conductance of an already open state.

The subconductance currents described in the present work meet the criteria for a true subconductance state of a single natural channel construct (Fox, 1987), and do not result from multiple channels of different conductance in the same patch, as has been suggested to result from the atypical assembly of subunits in expression systems (Gibb *et al.*, 1990). We have used native receptors and thus have avoided the controversies attendant on expression systems (Angelotti & Macdonald, 1993; Ebert *et al.*, 1996; Boileau *et al.*, 2003). When it occurs, the presence of more than one channel in the patch is easily detected from the obvious doubling of amplitude when full conductance currents coincide. In our records, interconversions between channel states can be seen occasionally (types 2,3 and 4 subconductances), both states can be seen consecutively as isolated currents, and the main state is not a multiple of the subconductance state, nor is the difference between the two seen as a conductance state of its own. The full and subconductance states are not additive, implying that they share a common current pathway. Lastly, full conductance states rise to their plateau current value virtually instantaneously between 80 and 90% of the time, and it strains credulity to hold that such currents stem from the chance, coincident openings of two independent channels. We feel confident in treating the lower conductance currents under study as an activated state of the nAChR channel, an alternative to the more widely studied full conductance state.

The relative abundance of subconductance currents in our system may be a reflection of the lower concentrations of agonist we employed, or it may be a characteristic of *Torpedo* receptors in liposomes. Certainly Tank *et al.* (1983) and Riquelme *et al.* (1990) both reported the appearance of subconductance currents in native *Torpedo* receptors reconstituted in giant liposomes and our own observations (unpublished) of *Torpedo* receptors expressed in oocytes confirm that subconductance currents are a significant component of the activated state profile.

Subconductance currents are evoked by both CCh and ACh and occur primarily as solitary events (type 1) belonging to a single stochastic population. This is consistent with the subconductance state being an autonomous entity with no obligatory connexion with the full conductance state, and where they do occur, complex combinations of full and subconductance currents are the result of chance coincidence. The relative dominance of the subconductance state at low agonist concentrations, and its proportional replacement by full conductance currents as concentrations rise is suggestive of a separate binding site with its own affinity for the ligands, and that the subconductance state may be a manifestation of a transit stage on the way to fuller agonist occupation of available binding sites or an independent state arising from a parallel activation pathway.

From curve fitting studies of endplate currents, Dionne *et al.* (1978) postulated that receptor activation was seen following the occupation of one or both of two binding sites on the receptor. Single channel studies revealed that full conductance channel open time distribution was described by the sum of two exponentials currents (Jackson, 1982; Jackson *et al.*, 1982) and the association of these two mean open times with the two sites proposed by Dionne followed when it was noted that the appearance of the longer state was concentration dependent (Colquhoun & Sakmann, 1981,1985; Labarca *et al.*, 1985). Despite early reservations about such a concept (Sine & Steinbach, 1984; Jackson, 1989), the principle has been accepted in many subsequent kinetic analyses of receptor activation, and the terms "monoliganded" and "diliganded" have become the argot of single channel kinetics (Lingle *et al.*, 1992; Edmonds *et al.*, 1995; Zhang *et al.*, 1995; Qin *et al.*, 1996; Salamone *et al.*, 1999; Akk & Auerbach, 1999; Grosman & Auerbach, 2001, 2002; Elenes & Auerbach, 2002; Colquhoun *et al.*, 2003; Chakrapani & Auerbach, 2004, 2005; Mitra *et al.*, 2005). This has left the subconductance current at best as an unexplained anomaly of trivial importance. Our data suggest that while it is true that the mean charge transfer by subconductance currents is small in comparison to that of the population of long full conductance currents (0.4 fC vs 12.7 fC), and therefore ignoring the subconductance is justified in terms of the physiologically significant activation of the receptor to cause end plate potential, the complete list of manifestations of receptor activation must include subconductance currents. Consequently, a full description of the activation sequence should take cognizance of this fact, though it is difficult to see how a two binding site model can accomplish this.

There has long been a dichotomy between biochemical binding data, with a K_D value in the range of 30nM for ACh, and functional data with EC_{50} or apparent K_D values between 100 and 400 μM and this has led to numerous speculations of other binding sites in addition to the pair of high affinity sites known to exist at the α-γ/δ/ε subunit interfaces (Brejc *et al.*, 2001; Grutter *et al.*, 2004). The number and location of such additional sites has remained uncertain (Dionne *et al.*, 1978; Dunn & Raftery, 1982a, b, 1983, 1993, 1997a, b, 2000; Raftery *et al.*, 1984; Strecker & Jackson, 1989). However, Dionne *et al.* conceded that two functional binding sites might only be a minimum and that there could well be more. That being so, there is no impediment to revisiting the activation schemes to incorporate a subconductance state.

Figure 2-1

Single channel currents recorded at different concentrations of ACh. Currents were recorded at an electrode potential of +90 mV and filtered at 2 kHz. Upward deflections from baseline represent openings. (A) Currents observed at 0.1 μM ACh. Both full conductance and subconductance currents (*) can be seen. Channel open durations for full conductance currents are brief. Short duration full conductance openings are marked by (O) and long duration full conductance openings marked by (X) (B) Currents observed at 1 μM ACh. Subconductance current (*) is visible among the full conductance currents in the burst. (C) Currents observed at 10 μM. Both short and long full conductance open durations are visible. (D) Currents observed at 100 μM. Long full conductance are plentiful, yet subconductance currents are clearly visible.

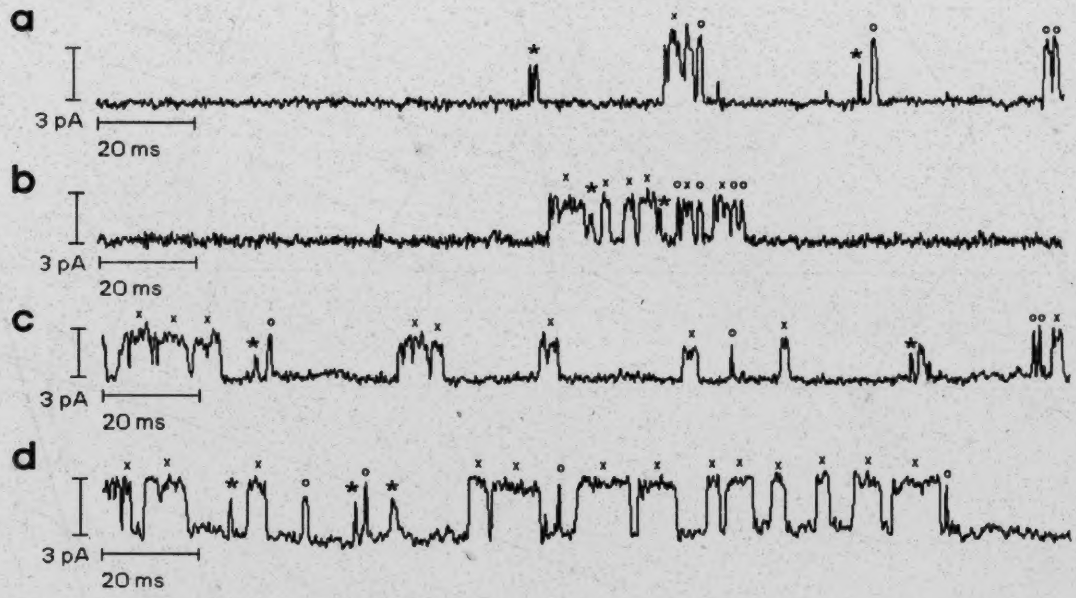

a
3 pA
20 ms
b
3 pA
20 ms
c
3 pA
20 ms
d
3 pA
20 ms

Figure 2-2

Examples of subconductance current types. (A) Type 1 - a single current rising from baseline and returning after a dwell time. (B) Type 2 - a complex waveform consisting of an initial subconductance current which rises after a dwell time to a full conductance current before returning to baseline. (C) Type 3 - a complex waveform consisting of a full conductance current which declines to a subconductance value before dropping to baseline after a dwell time. The opposite sequence to a Type 2. (D) Type 4 - a complex waveform in which a subconductance current is interposed between two full conductance currents without a return to baseline between them. Example shown is unusually long but provides clear visibility of the waveform.

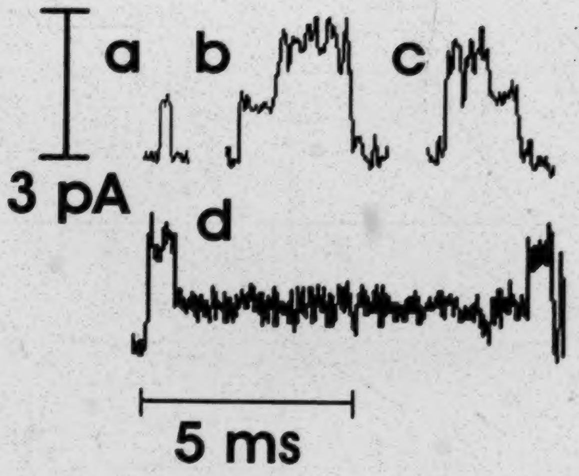

a
b
c
d
3 pA
5 ms

Figure 2-3

Relative frequencies of subconductance current types. At all concentrations measured, Type 1 currents are in the overwhelming majority. Type 4 show a slight increase with concentration, but this marginal. Data is cumulative from a minimum of 3 patches for each concentration.

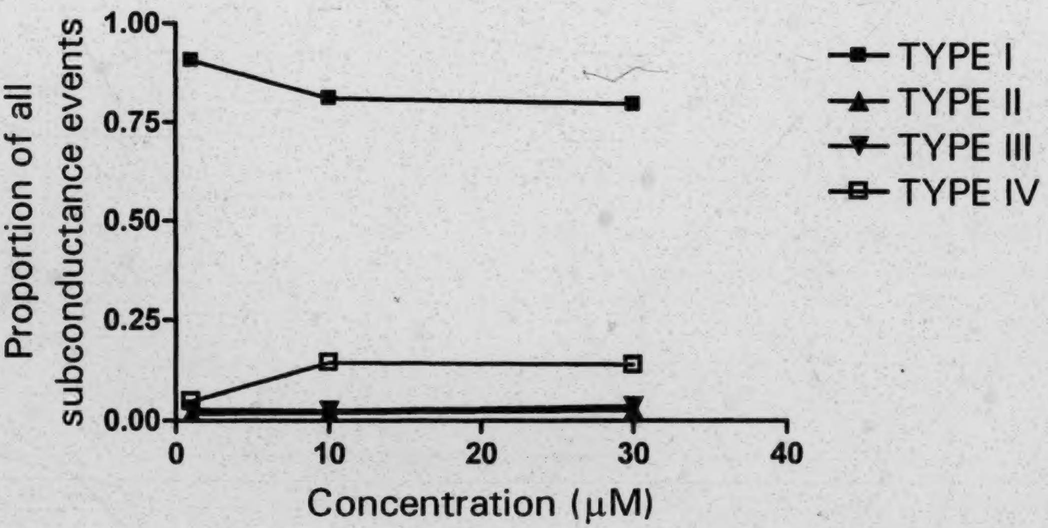

Proportion of all subconductance events
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
Concentration (μM)
TYPE I
TYPE II
TYPE III
TYPE IV

Figure 2-4

Amplitude histograms of currents evoked by agonists. (A) Currents evoked by 10 μM ACh. Two distinct populations of currents are clearly visible. The mean subconductance amplitude was 1.75 ± 0.22 pA and the mean full conductance amplitude was 2.7 ± 0.2 pA. Full conductance currents accounted for 56% of all currents. (B) Currents evoked by 10 μM CCh. Similar to ACh, two populations of currents were visible, with mean subconductance current amplitude 1.74 ± 0.18 pA and 2.77 ± 0.27 pA for the mean full conductance current amplitude.

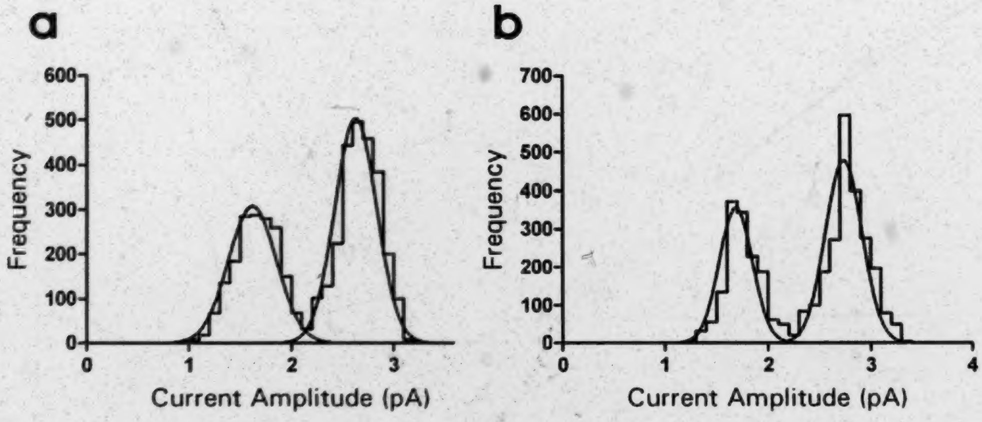

a
b
Frequency
600
500
400
300
200
100
0
700
Current Amplitude (pA)
0
1
2
3
4

Figure 2-5

Open duration analysis of currents evoked by ACh. (A) Sigworth-Sine plot of full conductance current durations with best fit curve resolved into two exponential populations. The mean short open duration time constant was 0.52 ± 0.02 ms and the mean long open duration time constant was 4.74 ± 0.05 ms. Long duration openings accounted for 41.7% of all full conductance openings. (B) Fraction of long duration openings as a function of ACh concentration. The rise in the proportion of long duration openings is linear over a concentration range of 5 orders of magnitude. (C) Sigworth-Sine plot of subconductance current durations with best fit curve resolved into a single exponential population. The mean open duration time was 0.43 ± 0.01 ms.

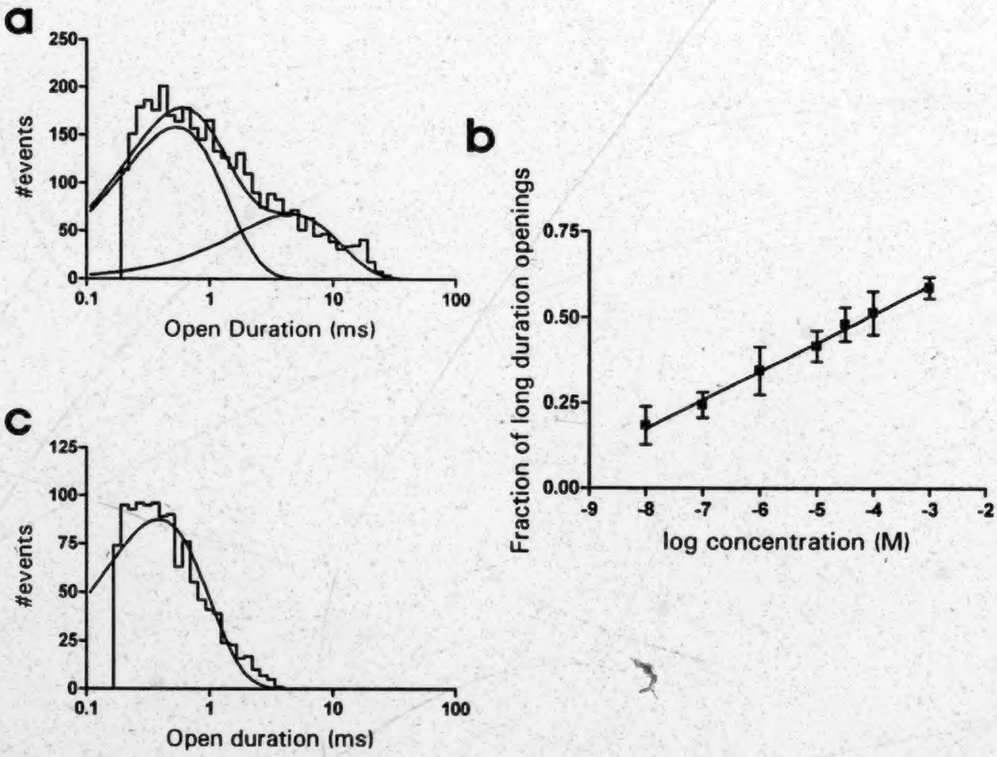

a
250
200
150
100
50
0
#events
0.1
1
10
100
Open Duration (ms)
b
Fraction of long duration openings
0.75
0.50
0.25
0.00
-9
-8
-7
-6
-5
-4
-3
-2
log concentration (M)
c
125
100
75
50
25
0
#events
0.1
1
10
100
Open duration (ms)

Figure 2-6

Stability of subconductance (a) and full conductance (b) time constants elicited by ACh. The average subconductance time constant was 0.43 ± 0.01 ms. For the full conductance open states, the shorter duration time constant (τ_S) was 0.52 ± 0.02 ms and the longer duration time constant (τ_L) was 4.74 ± 0.05 ms. Throughout the concentration range tested, the subconductance open duration time constants do not differ significantly ($P>0.05$) from one another. The fact that the time constants do not change over the concentration range suggests that channel block does not affect the channel at these concentrations. Furthermore, the long duration open time constants are concentration-independent, therefore they may be the result of multiple binding steps, with a higher occupancy resulting in a receptor that is stabilized in the open state for a longer duration.

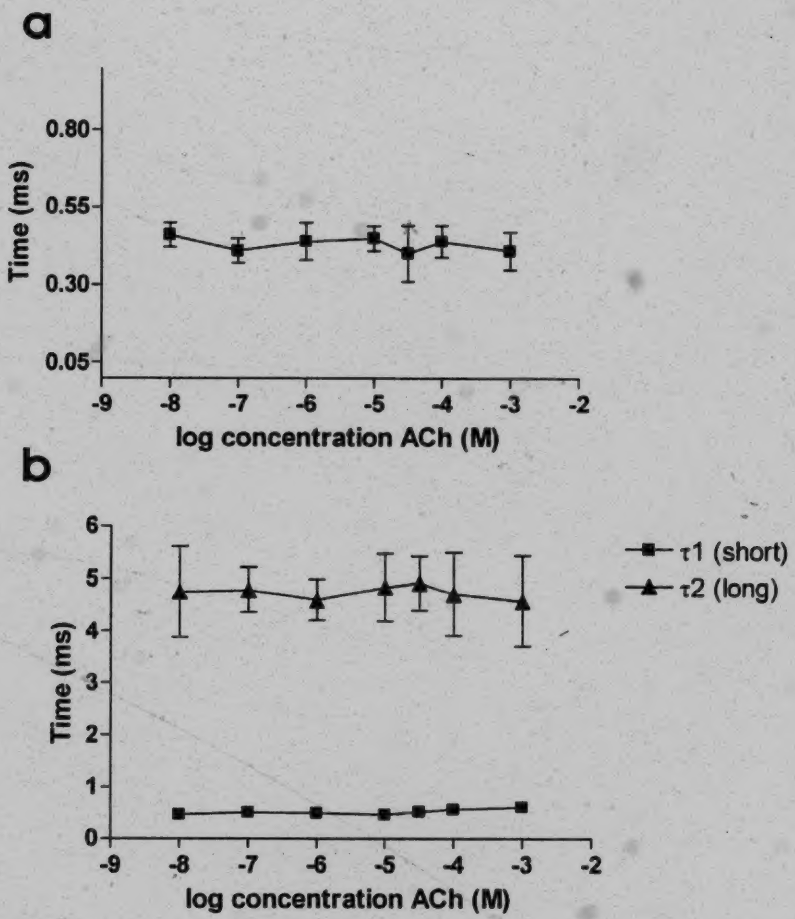
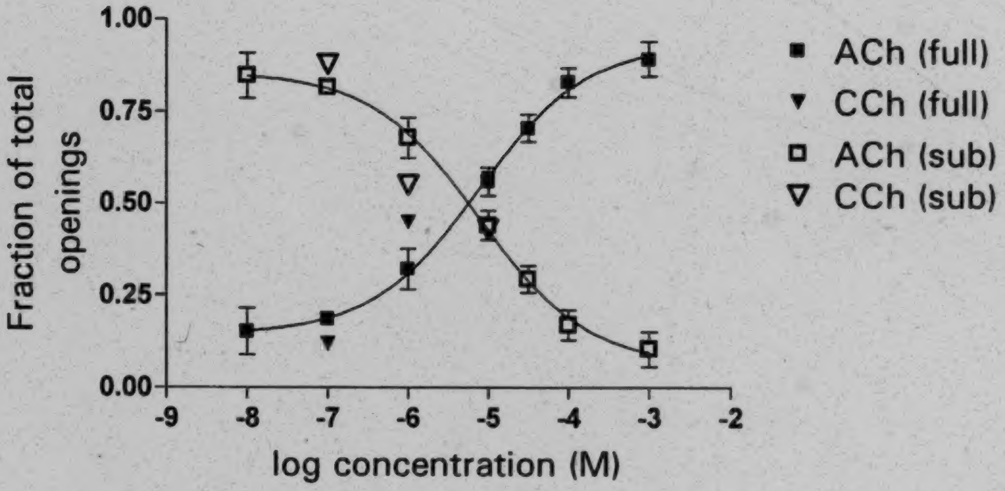

a
Time (ms)
0.80
0.55
0.30
0.05
-9
-8
-7
-6
-5
-4
-3
-2
log concentration ACh (M)
b
Time (ms)
6
5
4
3
2
1
0
-9
-8
-7
-6
-5
-4
-3
-2
log concentration ACh (M)
τ1 (short)
τ2 (long)

Figure 2-7

Full conductance currents as a fraction of all currents (solid symbols) and subconductance currents as a fraction of all currents (outline symbols). Even at 10^{-8} M ACh, full conductance currents accounted for more than 10% of all currents measured, while at 10^{-3} M 10% of all currents were subconductances. At least 3 patches were used to obtain each data point. The concentration-effect curve for CCh was virtually superimposable.



Fraction of total openings
1.00
0.75
0.50
0.25
0.00
-9
-8
-7
-6
-5
-4
-3
-2
log concentration (M)
■ ACh (full)
▼ CCh (full)
□ ACh (sub)
▽ CCh (sub)

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CHAPTER 3

Activation of the nicotinic acetylcholine receptor by semi-rigid agonists

Introduction

Although the ability of the nicotinic acetylcholine receptor (nAChR) to adopt more than one open conductance amplitude was first reported by Hamill & Sakmann (1981), the exact nature of this property has remained elusive. Poor signal to noise ratio, filtering of brief events, and the infrequency of the subconductance currents have all contributed to the relative obscurity of this activated state of the receptor. Despite these challenges, subconductance currents have been observed in many experimental preparations including rat myoballs (Hamill & Sakmann, 1981), embryonic chick myotubes (Auerbach & Sachs, 1983, 1984), *Xenopus* myotubes (Brehm *et al.*, 1984), frog muscle end-plate (Colquhoun & Sakmann, 1985), mouse skeletal muscle (Morris & Montpetit, 1986), and reconstituted receptors from *Torpedo* electroplax (Tank *et al.*, 1983; Riquelme *et al.*, 1990). Multiple-conductance states are not unique to the nAChR. Numerous conductance states have been reported for other ligand gated ion channels (Hamill *et al.*, 1983; Bormann & Clapham, 1985; Cull-Candy & Usowicz, 1987; Jahr & Stevens, 1987; Usowicz *et al.*, 1989; Bormann *et al.*, 1993).

We are particularly interested in the association between subconductance currents and semi-rigid agonists. These compounds generally possess ring structures that reduce the potential for flexion of the molecule. Early observations that tubocurarine, rather than acting as an antagonist, was in fact a partial agonist (Ziskind & Dennis, 1978; Jackson *et al.*, 1982; Trautmann, 1982; Morris *et al.*, 1983) and that it elicited subconductance currents (Trautmann, 1982; Takeda & Trautmann, 1984; Morris & Montpetit, 1986; Strecker & Jackson, 1989) led to the formation of two general hypotheses to describe these findings. One theory proposed that curare elicited full conductance events and that the observed subconductance openings were the result of partial block of the already open channel (Takeda & Trautmann; 1984). An alternative hypothesis proposed by Hamill & Sakmann (1981) suggested that the subconductances were a consequence of a variant or reduced conformational change resulting in a lower open conductance. Kawai *et al.* (2000) reported that the semi-rigid agonist methylarecolone elicited mainly subconductance openings despite the fact that it had a reported potency 3-fold greater than that of carbamylcholine. The low concentration used (2 μM) would seem to eliminate partial channel block as an explanation for the high frequency of subconductances and it was suggested that the lack of flexion within the molecule might limit conformational change within the receptor.

We have extended these earlier observations on methylarecolone to other semi-rigid agonists, specifically 1,1-dimethyl-4-phenylpiperazinium (DMPP) and phenytrimethylammonium (PTMA) with a view to determining the relationships between the activated states of the receptor evoked by these agonists. Furthermore, we have examined the effects of the novel semi-rigid ligand bis(N,N,-dimethylpiperidinyl) oxalate (DK1) on the single channel behaviour of the nAChR. These experiments were conducted with the aim of further elucidating the nature of the subconductance state through the use of nicotinic agonists that have more limited bond flexion and greater steric hindrance than the endogenous ligand acetylcholine. We have found that these compounds do not prevent the entrance into the full conductance state and that this state may possess two kinetically distinct open durations when openings are elicited by DK1. Furthermore, we postulate that the subconductance and full conductance open states are the result of independent binding sites on the receptor.

Materials and Methods

The materials and methods used in Chapter 3 are identical as those previously reported in Chapter 2 except where described in the following section.

Single channel analysis

The frequency of open events of the two conductance states were manually counted from at least ten consecutive 500 ms sections from 3 patches or more, starting with the first occurrence of currents. Only sections containing one or more openings within the time frame were included. Generally speaking, most of the sections that contributed to the data were taken immediately after seal formation so as to minimize the influence of desensitization at higher agonist concentrations.

Synthesis of bis(N,N,-dimethylpiperidinyl) oxalate (DK1)

The procedure used for synthesizing DK1 was modified from that used to synthesize protonated tertiary amine chlorides from diacyl dichlorides and N,N-dimethylethanolamine (Dunn & Raftery, 1997). One equivalent of the oxalyl dichloride (2 g; Sigma-Aldrich Canada, Oakville, ON) was dissolved in ~30 mL dichloromethane (Fisher Scientific Canada, Edmonton, AB) in a roundbottom flask. Excess 4-hydroxy-methylpiperidine (2 equivalents + 5% excess; Sigma-Aldrich Canada, Oakville, ON) was added, creating a pale yellow precipitate. The solution and precipitate were refluxed for 3 hours at ~40°C after which the precipitate was filtered three times and washed with dichloromethane. The precipitate was dried *in vacuo* in the presence of NaOH and phosphorus pentoxide in separate crucibles overnight. The dried precipitate was dissolved in ~50 mL distilled water and titrated to pH 10 with 1 M NaOH. The dissolved precipitate was extracted three times with ~50 mL dichloromethane in a 250 mL separatory funnel. The organic layer was collected from the extraction and dried over anhydrous magnesium sulphate overnight. To further remove dissolved impurities, the collected organic layer was evaporated in a Roto-Vap at ~40°C. After the solvent was totally evaporated, a yellow oil remained in the bottom of the flask. This oil, containing a tertiary amine, was reacted with 2 equivalents + 5% excess methyliodide to form the charged quaternary nitrogen. Upon reaction, a fine white precipitate, the final product, was formed that was subsequently washed and filtered 3 times with cold dichloromethane. The product was dried and stored in an airtight glass container at room temperature for future use.

Solutions and drugs

All electrolytes and non-aqueous solvents were purchased from Fisher Scientific Canada (Edmonton, AB). Acetylcholine chloride, 1,1-dimethyl-4-phenylpiperazinium iodide, and phenyltrimethylammonium chloride were purchased from Sigma-Aldrich Canada (Oakville, ON). The molecular structures for these compounds are represented in Figure 3-1.

Results

Amplitude

Sample single channel current recordings from ACh, DMPP, and PTMA at various concentrations are shown in Figure 3-2. Two open amplitudes are clearly visible in the traces, a full conductance open state and a lesser, or subconductance, open state marked by (*). In the presence of acetylcholine, the receptor produces two conductance states (2.70 ± 0.20 pA and 1.75 ± 0.22 pA). Similar amplitudes were observed for DMPP (2.66 ± 0.29 pA, 1.79 ± 0.25 pA) and PTMA (2.79 ± 0.33 pA, 1.79 ± 0.29 pA). Sample amplitude histograms for DMPP from which the mean amplitudes were calculated appear in Figure 3-3. The mean chord conductance values (30.1 ± 0.8 pS and 19.1 ± 0.6 pS) were calculated from the mean amplitudes of the histograms from each of the three agonists used. Similar conductance values for reconstituted nicotinic receptors have been reported by Tank *et al.* (1983), Suarez-Isla *et al.* (1983), and Labarca *et al.* (1984).

Figure 3-4 shows the proportion of full conductance currents as a fraction of the total number of open events. Compared to ACh, the semi-rigid agonists elicit a lower full conductance fraction at all equimolar concentrations and, in fact, never reach the level of ACh activation at the highest concentration we tested. The curve for these agonists is right-shifted ~2 orders of magnitude. Whereas ACh produced currents at concentrations as low as 10 nM, the lowest concentration of semi-rigid agonist at which we could reliably analyze currents was 10 μM. At this minimal concentration, both DMPP and PTMA elicited ~10% full conductance events which is approximately 5.5 fold lower than that observed for ACh (56 ± 4%). At the highest concentrations of the semi-rigid agonists tested (3 mM), we found that DMPP and PTMA elicited 63.3 ± 7.8% and 71.5 ± 6.9% full conductance events, respectively. Both semi-rigid agonists appear to approach the same proportion of full conductance openings that was measured for ACh, however a plateau for the semi-rigid agonists was not observed because at concentrations > 3 mM, significant channel block occurred and the electronic balancing of the amplifier was not possible.

Frequency of currents

The number of openings, both subconductance and full conductance, observed within successive 500 ms bins were counted and plotted for ACh and DMPP. As might be expected, increasing concentrations of ACh elicited a greater number of openings, both of full and subconductance currents. At lower concentrations of ACh (<1 μM), the number of subconductance events was greater than full conductance events with 10 nM ACh eliciting 0.69 ± 0.45 full conductance events/bin and 3.85 ± 0.21 subconductance events/bin (figure 3-5a). The number of subconductance openings remained relatively constant until a concentration of 10 μM was reached, at which point the number of openings increased to 11.77 ± 1.04 events/bin. Evoked subconductance openings appeared to reach a maximum frequency at 100 μM ACh where the number of observed events was 14.27 ± 0.87 events/bin. The calculated EC_{50} for this curve was 5.4 ± 1.6 μM and the Hill slope was 1.0.

The pattern of full conductance openings followed a trend similar to that seen with subconductance openings, however the frequency of openings was much greater for the full events at higher concentrations compared with that of the subconductances (Figure 3-5b). Subconductance openings form the majority of observed events up to a concentration of 10 μM ACh, at which point the rate of full conductance openings is greater (16.72 ± 1.72 events/bin). Increasing the concentration of ACh beyond 10 μM produced further increases in the frequency of full conductance openings up to 1 mM (81.68 ± 9.43 events/bin), an approximately five-fold greater number of full conductance openings vs subconductances at the same concentration. An EC_{50} of 39.6 ± 1.2 μM and a Hill slope of 1.4 was obtained which is similar to that observed in nAChR expressed in *Xenopus* oocytes (EC_{50} = 24.3 μM, Hill slope = 1.6 ± 0.1) (Kapur *et al.*, 2006) or flux studies (Raftery *et al.*, 1983).

Analysis of the frequency of currents evoked by DMPP is shown in Figure 3-5c. Enumeration of subconductance openings elicited by DMPP produced a curve that is superimposable on that for ACh subconductance currents. For DMPP at concentrations ranging from 10 µM to 3 mM, the frequency of subconductance openings remained relatively stable at ~10-14 events/bin. Below 10 µM DMPP, no currents were observed (n=2 patches, 1 µM DMPP). The frequency of full conductance currents increased in a concentration-dependent manner, from 1.24 ± 0.17 events/bin at 10 µM to 24.50 ± 2.09 events/bin at 3 mM. No plateau was reached. Since we could not record concentrations > 3 mM DMPP, at which point the current frequency was still sub-maximal, we could not calculate an EC_{50} for the semi-rigid agonist full conductance curve.

Open channel durations

Examination of the open duration distributions of the currents evoked by the semi-rigid agonists produced results similar to those obtained when ACh was the agonist (unpublished observations). Figures 3-6a&b present sample Sigworth-Sine plots of subconductance openings elicited by DMPP at concentrations of 10 µM and 1 mM. The average time constant of all subconductance open time constants was 0.62 ± 0.02 ms and was unaffected by concentration. The time constants for PTMA at the same concentrations were similar, with an average τ-value of 0.52 ± 0.02 ms. The two average time constants were significantly different (P=0.0042), however the absolute difference (0.10 ms) was small. Both DMPP (P=0.0001) and PTMA (P=0.0022) have mean subconductance open durations that were significantly longer than that observed for ACh (τ=0.43 ± 0.01 ms).

Full conductance currents evoked by DMPP and PTMA fell into two distinct open duration populations, corresponding well with previously reported biphasic population distributions in systems using reconstituted receptors (Tank *et al.*, 1983; Labarca *et al.*, 1984; Riquelme *et al.*, 1990). Sample Sigworth-Sine plots for full conductance currents elicited by DMPP at 100 μM and 1 mM are shown in Figures 3-6c&d. The means of the two open duration populations were separated by > 3 ms at all concentrations tested. The mean open durations of the two populations for DMPP were τ_S=0.56 ± 0.02 ms and τ_L=4.17 ± 0.24 ms, where τ_S represents the short duration time constant and τ_L represents the long duration time constant. For PTMA, the mean open durations were τ_S=0.51 ± 0.02 ms and τ_L=3.42 ± 0.29 ms. The two time constants were independent of agonist concentration. There was no significant difference between the time constants for the two semi-rigid agonists (both τ_S and τ_L) and the time constants remained stable throughout the concentration range tested for both subconductance and full conductance openings (Figures 3-7 & 3-8). When compared to the short time constant for ACh (τ_S=0.52 ± 0.02 ms), the time constant for DMPP is significantly longer (P=0.047) whereas that for PTMA was not significantly different (P>0.05) from that of ACh. The long time constant for ACh (τ_L=4.74 ± 0.05 ms; see Chapter 2) was significantly greater than that of DMPP (P=0.0073) and PTMA (P=0.0002).

We next examined the effect of concentration on the fraction of the area contributed by long duration full conductance openings (Figure 3-9). We previously found that long duration openings increased in frequency with increasing concentrations of ACh. At the lowest concentration of ACh (10 nM), 18.38 ± 5.61% of all full conductance events were classified as long openings, whereas 1 mM elicited 58.75 ±

3.08% long duration openings. This contrasts with the semi-rigid agonists which produced a minority of long duration openings at all concentrations studied. DMPP 100 μM produced 18.28 ± 5.61% which increased slightly to 23.56 ± 4.10% at a concentration of 3 mM. For PTMA, the results were similar, with 30 μM eliciting 23.21 ± 5.11% and increasing to 38.92 ± 4.54% at 3 mM. The fractions of long duration openings produced by the two semi-rigid agonists were not significantly different at all concentrations except 3 mM (P=0.026). Lower concentrations elicited too few full conductance events for proper open duration analysis.

Novel semi-rigid agonist, DK1

The structure of the novel semi-rigid agonist, bis(N,N,-dimethylpiperidinyl) oxalate (DK1), is different from those semi-rigid molecules previously described in that it possesses two quaternary nitrogens located within piperidine rings and separated by an interonium distance of 11.80 Å (Figure 3-10). The unique structure of DK1 also produced a unique pattern of activation. Sample single channel recordings elicited by DK1 are shown in Figure 3-11. The range of concentrations tested for DK1, 10 μM to 3 mM, was chosen because any concentration lower than 10 μM produced too few openings and concentrations above 3 mM produced significant channel block and problems of ionic imbalance. The sweep of these sample recordings is much longer than that previously shown for the other agonists (1000 ms vs. 200 ms) as there were few openings at even the highest concentration tested (3 mM). What is immediately evident from these recordings is that there are far fewer openings (both subconductance and full conductance) than the other agonists tested, the vast majority of these openings are subconductance openings, and there are both short and long duration subconductance openings.

When examining open amplitude histograms derived from single channel recordings elicited by DK1, it is apparent that the subconductance amplitude (1.72 ± 0.39 pA) dominates at all concentrations, a sample of which is presented in Figure 3-12. The few full conductance openings that were recorded fell within a similar distribution to that observed with other agonists (2.77 ± 0.64 pA). The point conductances for these amplitudes were within error for those calculated for ACh and DMPP/PTMA as would be expected if DK1 were to activate the receptor through a similar mechanism. The proportion of full conductance currents as a fraction of all open events is represented in Figure 3-13. Even at the highest concentration of DK1 (3 mM), the fraction of full conductance openings is lower (10.1%) than that of the lowest concentration of ACh tested (12.0%). Since higher concentrations of DK1 were not technically possible for reasons described previously, no plateau for the curve was reached and it is impossible to determine if DK1 can elicit full conductance openings with equal frequency to ACh if a high enough concentration could be reached. It is clear, however, that the full conductance fraction curve is dramatically shifted to the right and a very high concentration of DK1 is required to elicit full conductance openings.

Duration analysis of openings elicited by DK1 reveals two distinct populations of subconductance open durations, a phenomenon not previously observed with either nicotinic acetylcholine receptor agonists. Sample open duration histograms for currents elicited by 10 μM and 1 mM DK1 are presented in Figure 3-14. While two populations of open durations are not immediately evident in the sample histogram for 10 μM DK1, at 1 mM DK1, two stochastic populations of open durations are clearly evident therefore a double exponential curve fit was imposed on the data for 10 μM DK1. The lower concentration of DK1, 10 μM, produced subconductance open duration time constants of τ_S=0.36 ± 0.05 ms and τ_L=7.03 ± 2.48 ms, while 1 mM DK1 produced comparable subconductance open duration time constants of τ_S=0.46 ± 0.06 ms and τ_L=5.95 ± 1.62 ms. The difference between the two long duration time constants was found to be non-significant ($P>0.05$) and was most likely due to the low frequency of long duration openings recorded at 10 μM DK1. The two open duration time constants remained stable throughout the concentration range tested, depicted in Figure 3-15, indicating that there was no channel block present nor was there a change in the mechanism of activation due to concentration.

When we examined the fraction of the area contributed by long duration subconductance openings, we found that the proportion of long duration openings did not increase significantly ($P>0.05$) from 10 μM to 3 mM DK1 (Figure 3-16). At the lowest concentration of DK1 (10 μM), 14.7 ± 5.2% of all subconductance openings were classified as long duration, while at the maximum concentration of DK1 tested (3 mM), 34.0 ± 7.0% of all subconductance openings were classified as long duration. Although there was no statistically significant concentration-dependent increase in the fraction of long duration openings, the strong trend of the data suggest that a significant difference would have been achieved if the use of higher concentrations had been possible. No similar analysis could be performed on full conductance openings as there were too few events at all concentrations tested. Furthermore, no in-depth analysis of the frequency of openings per unit time could be performed as there were too few openings within the specified 500 ms bin width to provide any meaningful data.

Discussion

Previous studies with tubocurarine produced conflicting theories of the origin of the subconductance state. Some proposed that the subconductance currents arose from a partially occluded channel pore (Takeda & Trautmann, 1984; Strecker & Jackson, 1989), while others suggested allosteric conformational changes as an explanation for the subconductance phenomenon (Hamill & Sakmann, 1981; Auerbach & Sachs, 1983; Morris & Montpetit, 1986). Evidence presented by both sides, however, has been equivocal and the investigation into the fundamental nature of the subconductance currents has lapsed since the early 1990s. The association between the reported frequency of subconductance currents evoked by semi-rigid agonists led to the suggestion that the flexibility of the molecule may be a factor in restraining the degree of receptor activation (Kawai *et al.*, 2000), and this has rekindled our interest in the subject.

The appearance of the subconductance state occurs at the lowest concentrations of semi-rigid agonists examined when the frequency of full conductances is quite low. At no time did we observe a patch that contained exclusively only subconductances or only full conductance currents leading us to conclude that the two open amplitudes are indeed activated states of the same receptor. Furthermore, the two open amplitudes are not multiples of a unitary current as might be expected if they were two separate channels; the subconductance current consisting of one channel and the observed full conductance current the coincidental opening of two discrete channels. It would seem highly improbable that two independent channels would open and close simultaneously with great frequency, especially at lower concentrations where channel activity was sparse. At high ACh concentrations where the channel had a very high probability of being open we saw no addition of the subconductance state to the already open full conductance state, thus confirming that the subconductance state was not a separate subtype of nicotinic receptor. These findings are in agreement with the criteria proposed by Fox (1987) to distinguish a true subconductance state from the possibility of multiple channels in a patch.

Analysis of the subconductance open durations revealed only a single exponential population with all agonists tested, contrasting with the two populations of open durations observed in the full conductance currents. Although this single kinetic state was short lived (~0.6 ms), the influence of different compounds on the mean open duration was apparent. The rank order of mean subconductance open duration was DMPP>PTMA>ACh. This is not surprising, as it has been shown that the open durations for the full conductance currents are dependent upon the agonist used (Katz & Miledi, 1973; Mathie *et al.*, 1991), and that this is mediated by alterations in the opening and closing rate constants or the dissociation rate constant (Akk & Auerbach, 1999). We might expect that the chemical structure of the compound plays a role in determining the duration of the open state if the subconductance open state is the direct result of a binding event between a ligand and a binding site. Differing association/dissociation rate constants or opening/closing kinetics of the subconductance state are, therefore, also to be expected with different chemical structures. The structural determinants of agonists which dictate channel open time are not easy to discern (Cooper *et al.*, 1996). However, it is noteworthy that the effect of an agonist on subconductance and full conductance open states need not be identical when compared to ACh. This speaks to a uniqueness between ligand and binding site and a separation between the binding sites associated with full conductance currents and those that evoke subconductance currents.

The most significant of our observations was the extent to which the concentration-frequency curve for individual currents evoked by DMPP was separated from that of ACh. Since the ACh and DMPP curves for subconductance currents are exactly superimposable, the principle difference lies in the concentration range over which the full conductance currents are evoked. Such a separation in the concentration dependence of the various responses is consistent with two independent systems for evoking the two amplitudes of current seen. The concentration dependence of the subconductance-related binding site seems little affected by agonist structure. However, the potency of the semi-rigid agonists at evoking full conductance currents is greatly reduced in comparison to ACh. It is this major loss in potency that confers the poor partial agonist classification on the semi-rigid compounds. The technical limitations of working with such high concentrations prevented us from determining if the concentration-frequency curve for semi-rigid-evoked full conductance currents would reach the maximum value of that for ACh or if channel block, which is to be expected at high concentrations (Sine & Steinbach, 1984; Ogden & Colquhoun, 1985) might intervene to limit intrinsic efficacy.

The novel nicotinic agonist DK1 produced results that were highly surprising when compared to those previously observed with either ACh or DMPP/PTMA. Since there has never been a compound similar in structure to DK1 reported to be tested in the literature, it was unknown what effects the ligand would produce. Originally, the purpose of synthesizing this class of compound was to examine the effects of varying the chain length between the two quaternary nitrogens. Although we synthesized a number of molecules ranging in chain length from 11.80 Å (DK1) to 20.67 Å (DK7), the only one to show any reproducible activation of the receptor was DK1.

When we examined the effect of DK1 on amplitude, we found that it produced an extremely low full conductance fraction (~10%) at concentrations as high as 3 mM. This was in stark contrast to the full conductance fractions observed for ACh at the maximal concentration tested (~90% at 1 mM) or that of the semi-rigid agonist DMPP (~63%). The fact that DK1 produces a very low full conductance fraction may be one of the reasons that the compound is a very poor agonist. Furthermore, the low frequency of openings, both subconductance and full conductance, most likely contributes to the partial agonist properties of DK1. Whole cell recordings from *Xenopus* oocytes failed to achieve a maximum current with a DK1 concentration of 10 mM, therefore no EC_{50} could be calculated although the amplitude of the current obtained at 10 mM DK1 was approximately 1/10 that obtained with ACh (Kula *et al.*, 2005). This presumably high EC_{50} at the whole cell level is predicted from the data obtained using single channel patch clamp techniques from its low frequency of channel openings and low full conductance fraction.

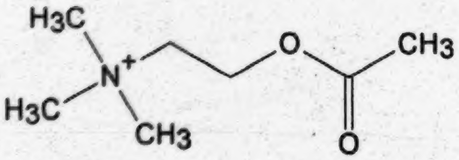
The most surprising finding from the experiments carried out with DK1 was the appearance of a second stochastic population of subconductance open duration which was previously unseen with other agonists. We have found that ACh and both DMPP and PTMA elicit a single population of subconductance open durations, the time constant of which is agonist-dependent. The time constants, although variable between agonists, remain unchanged with concentration. Furthermore, a second, longer open duration population of subconductance currents does not emerge as the concentration is increased as was observed with full conductance openings. DK1, on the other hand, produced two subconductance open durations with time constants of approximately 0.4 ms and 6.5 ms. Furthermore, the fraction of the longer duration openings increases with concentration. As this was not observed with other agonists, an explanation as to its meaning is difficult. With full conductance openings, it was suggested that the two open durations were the result of the occupation of either one or both agonists binding sites (Labarca *et al.*, 1984, 1985). It is tempting to ascribe a similar mechanism to the two subconductance open durations elicited by DK1, however we did not observe such a phenomenon with either ACh or the semi-rigid agonists DMPP and PTMA. Perhaps there are indeed two populations of subconductance open durations with all agonists but the shorter duration time constant is smaller than the detection threshold of 200 μs and the time constant that we measured was the longer one. Another possibility might be that the time constants produced by the other agonists are very similar, and therefore cannot be distinguished from one another. DK1, on the other hand, may stabilize the receptor in the longer subconductance open duration, thus making it possible to distinguish the two populations. Yet another possibility might be that there are two possible conformations that DK1 can adopt within the binding pocket which produce two distinct subconductance open durations. One of the two conformations may stabilize the receptor in the open state for a longer period than the other conformation, thus creating two open durations which are independent of the occupancy of either one or both classical binding sites.

From the experimental evidence obtained thus far, it is impossible to determine which of the possibilities is correct and further data are required. Recordings performed with a higher filter frequency (>10 kHz) using ACh and DMPP/PTMA may be able to discern a second, extremely short, subconductance open duration. Channel open durations have been reported to be as short as 5 μs (Hallermann *et al.*, 2005), therefore further investigation with greater sensitivity toward short duration subconductance openings may confirm the observations of two open duration populations elicited by DK1. Although the exact nature of the second population of subconductance open durations is as yet unresolved, the fact that the subconductance state may contain two populations of open durations and that the proportions of the two populations are concentration dependent warrants further investigation and incorporation into current kinetic schemes of the nicotinic acetylcholine receptor.

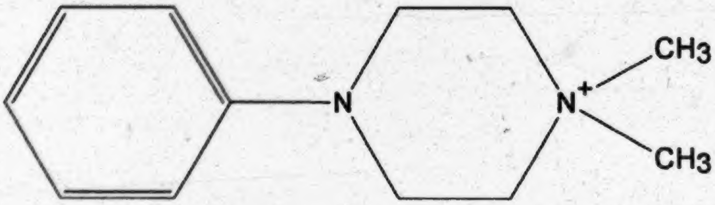
In summary, therefore, there appear to be independent binding sites capable of evoking either subconductance or full conductance currents, and the individual kinetic parameters for agonists at each site determine the overall classification of the compound involved. Furthermore, the kinetic scheme required to explain the subconductance state is most likely much more complex than previously considered when agonists such as ACh or DMPP were used. The requirement to add another active state, the long open duration subconductance, should be addressed and an investigation into whether this state exists with other agonists should be investigated.

Figure 3-1

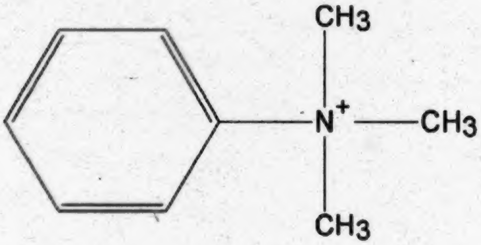
Chemical structures for acetylcholine (ACh), 1,1-dimethyl-4-phenylpiperazinium (DMPP), and phenyltrimethylammonium (PTMA)



ACh



DMPP



PTMA

Figure 3-2

Sample single channel recordings evoked by ACh, DMPP, and PTMA. Currents were recorded at an electrode potential of +90 mV and filtered at 2 kHz. Upward deflections from baseline represent channel openings. Openings marked by (*) indicate subconductance currents. (A) Currents elicited by 10 μM ACh. Subconductance currents are evident, as are two open duration populations of full conductance currents. (B) Currents evoked by 10 μM DMPP. Few currents are visible at this concentration, the majority of which are subconductance currents. Most full conductance currents are short in duration. (C) Currents evoked by 3 mM DMPP. Channels open more frequently at this concentration, the majority of which are full conductance currents. (D) Currents elicited by 10 μM PTMA. Results are similar to DMPP at the same concentration. (E) Currents elicited by 3 mM PTMA. Activation of the receptor resembles that seen at an equimolar concentration of DMPP.

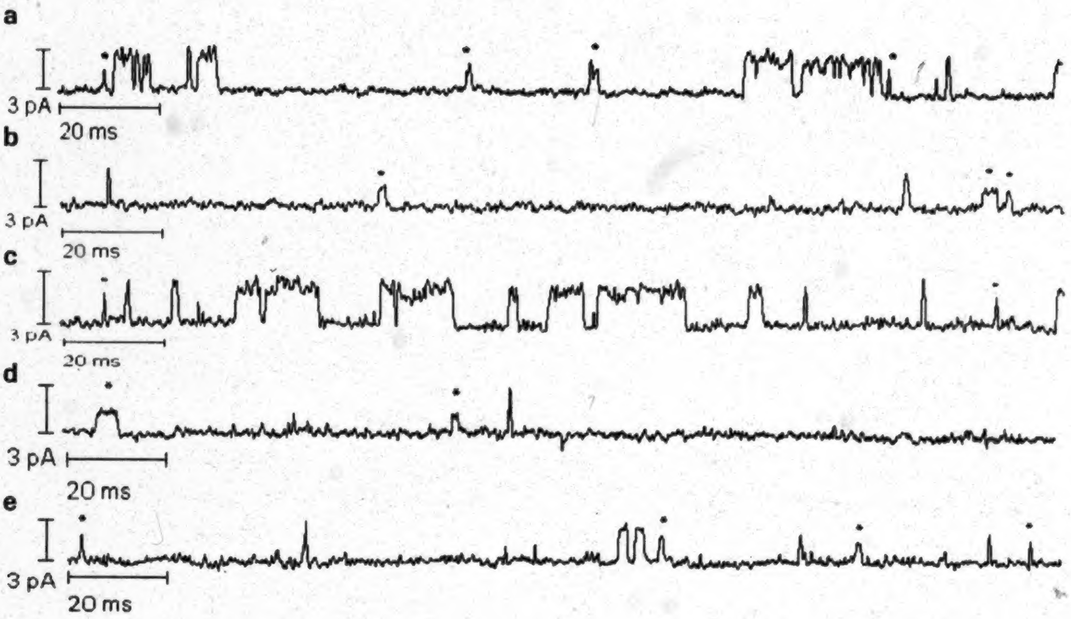

a
3 pA
20 ms
b
3 pA
20 ms
c
3 pA
20 ms
d
3 pA
20 ms
e
3 pA
20 ms

Figure 3-3

Sample amplitude histograms for currents evoked by DMPP. (A) Amplitude histogram for currents elicited by 10 μM DMPP from a single patch. The mean amplitudes for the two open states are 1.83 ± 0.28 pA (s.d.) and 2.69 ± 0.25 pA (s.d.). The vast majority of events are subconductance openings (~90%). (B) Amplitude histogram for currents elicited by 3 mM DMPP from a single patch. The mean amplitudes for the subconductance and full conductance currents are 1.76 ± 0.34 pA (s.d.) and 2.78 ± 0.33 pA (s.d), respectively. At this concentration, ~63% of all recorded events are full conductance openings.

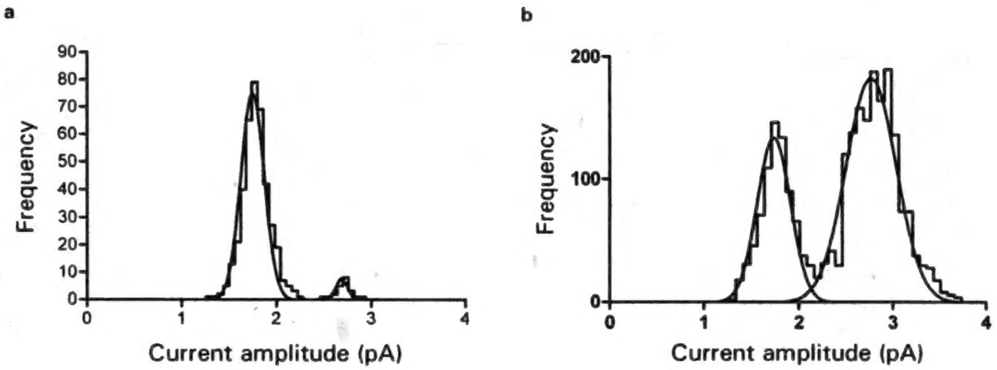

a
90
80
70
60
50
40
30
20
10
0
Frequency
0
1
2
3
4
Current amplitude (pA)
b
200
100
0
Frequency
0
1
2
3
4
Current amplitude (pA)

Figure 3-4

Full conductance currents as a fraction of all observed open events. The curves for the semi-rigid agonists DMPP and PTMA are right-shifted ~2 orders of magnitude from that for ACh. At the lowest concentrations of all agonists used, full conductance currents account for ~10% of all currents. Higher concentrations increase the full conductance fraction with all agonists; however the fraction rises much faster with ACh than with either DMPP or PTMA. Furthermore, the highest concentrations of DMPP and PTMA elicit a full conductance fraction of ~65% which is much lower than that observed for ACh at 1 mM (~90%).

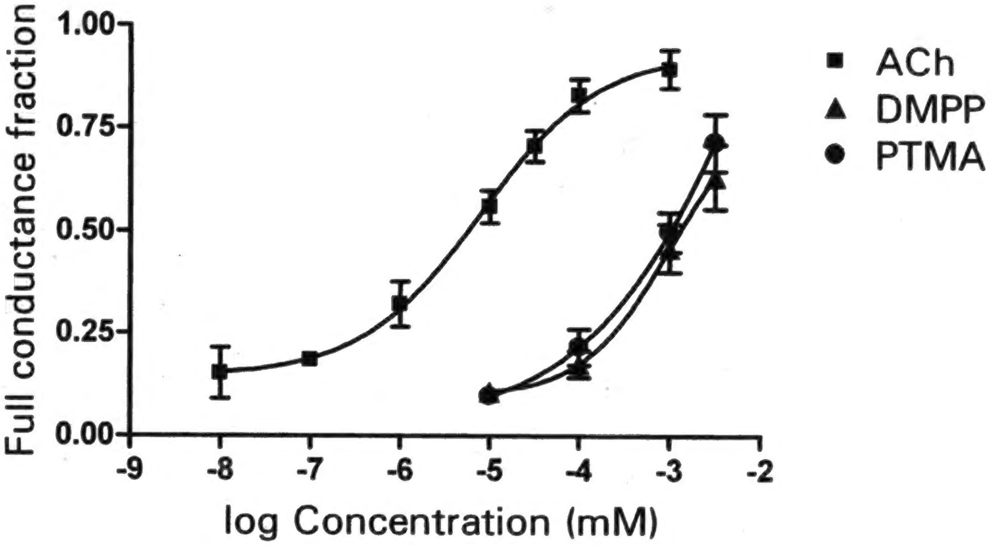

Full conductance fraction
1.00
0.75
0.50
0.25
0.00
-9
-8
-7
-6
-5
-4
-3
-2
log Concentration (mM)
■ ACh
▲ DMPP
● PTMA

Figure 3-5

Concentration-frequency relationship currents/500 ms evoked by ACh and DMPP. Subconductance currents (a) evoked by ACh rise to reach their maximum over a concentration range of 1-100 μM. The EC_{50} for the subconductance curve was 5.4 ± 1.6 μM and the Hill slope was 1.0. The full conductance current frequency (b) rises to a much greater level than the subconductance curve over the concentration range 1-300 μM. The EC_{50} for the full conductance curve was 39.6 ± 1.2 μM and the Hill slope was 1.4. The concentration-frequency curve for subconductance currents evoked by DMPP (c) was superimposable on that for ACh subconductance currents. However, the concentration-frequency curve for DMPP-evoked full conductance currents did not leave baseline until a concentration of 100 μM. Results from concentrations greater than 3 mM could not be obtained due to technical limitations of the recording system.

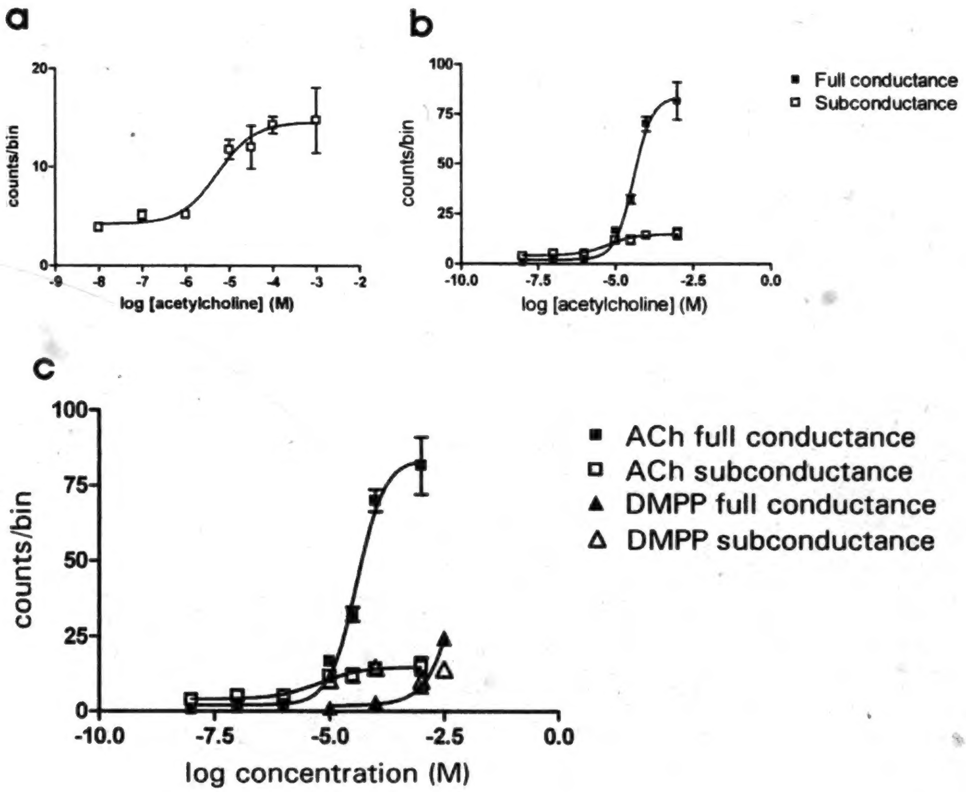

a
20
10
0
counts/bin
-9
-8
-7
-6
-5
-4
-3
-2
log [acetylcholine] (M)
b
100
75
50
25
0
counts/bin
-10.0
-7.5
-5.0
-2.5
0.0
log [acetylcholine] (M)
■ Full conductance
□ Subconductance
c
100
75
50
25
0
counts/bin
-10.0
-7.5
-5.0
-2.5
0.0
log concentration (M)
■ ACh full conductance
□ ACh subconductance
▲ DMPP full conductance
△ DMPP subconductance

Figure 3-6

Open duration analysis of currents evoked by DMPP. All Sigworth-Sine plots are constructed from a single patch. (A) Sigworth-Sine plot of subconductance currents evoked by 10 μM DMPP. A single exponential population is evident with a time constant of 0.70 ± 0.05 ms. (B) Sigworth-Sine plot of subconductance currents evoked by 1 mM DMPP. This concentration produced a similar time constant (0.59 ± 0.08 ms). (C) Full conductance open durations evoked by 100 μM DMPP represented as a Sigworth-Sine plot. Two open duration populations are evident. The short duration time constant (τ_S) was 0.60 ± 0.05 ms and the long duration time constant (τ_L) was 3.88 ± 0.51 ms. The area contributed by the full conductance long duration openings was 16.4%. (D) Full conductance open durations evoked by 1mM DMPP represented as a Sigworth-Sine plot. The two time constants were τ_S=0.53 ± 0.03 ms and τ_L=4.00 ± 0.22 ms. The long duration full conductance openings proportion was slightly higher (25.1%).

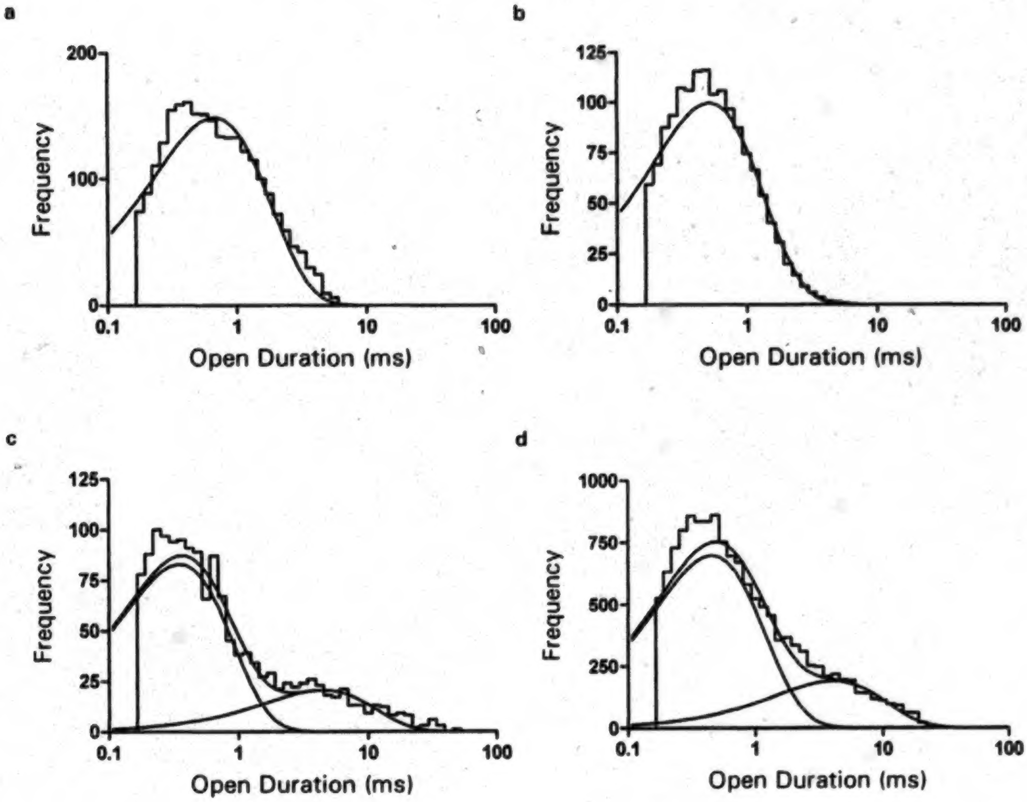

a
b
c
d
Frequency
Open Duration (ms)
200
100
0
125
100
75
50
25
0
1000
750
500
250
0
0.1
1
10
100

Figure 3-7

Stability of subconductance time constants elicited by DMPP and PTMA. Throughout the concentration range tested, the subconductance open duration time constants do not differ significantly ($P>0.05$) from one another for each agonist. This suggests that the same mechanism of activation occurs throughout the concentration and that there is no significant channel block.

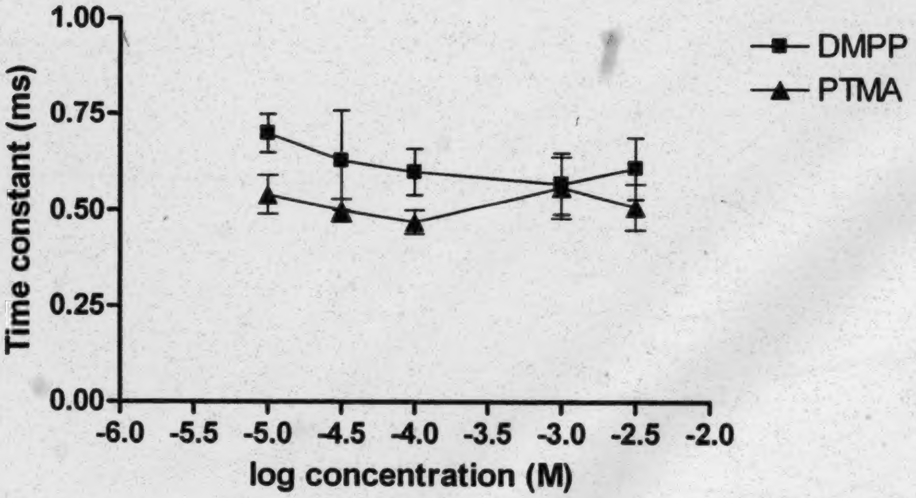
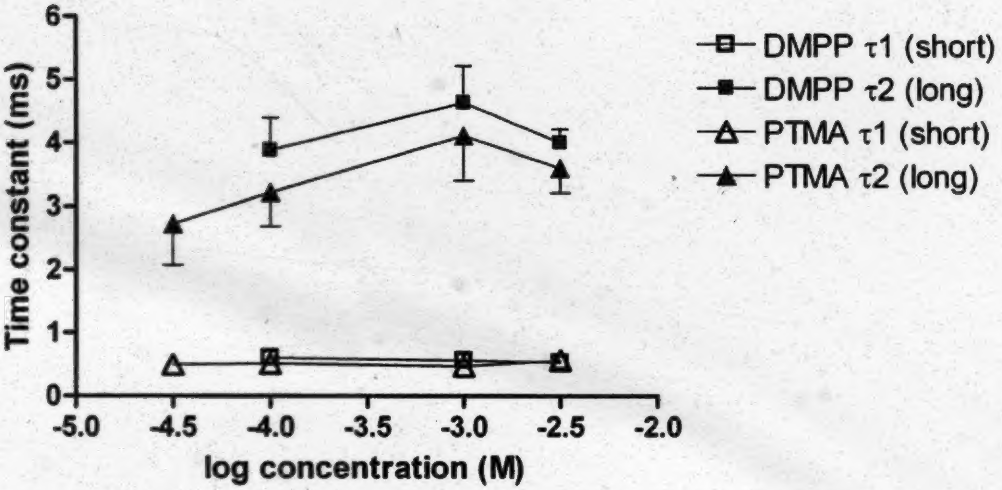

1.00
0.75
0.50
0.25
0.00
Time constant (ms)
-6.0
-5.5
-5.0
-4.5
-4.0
-3.5
-3.0
-2.5
-2.0
log concentration (M)
DMPP
PTMA

Figure 3-8

Stability of full conductance time constants elicited by DMPP and PTMA. For both agonists and both populations of open times, there is no significant difference ($P>0.05$) between the time constants measured over the entire concentration range.



Time constant (ms)
0
1
2
3
4
5
6
-5.0
-4.5
-4.0
-3.5
-3.0
-2.5
-2.0
log concentration (M)
DMPP τ1 (short)
DMPP τ2 (long)
PTMA τ1 (short)
PTMA τ2 (long)

Figure 3-9

Fraction of long duration openings as a function of agonist concentration. Linear increase in fraction of long duration openings for all three agonists, however best-fit lines for DMPP and PTMA are right-shifted ~3 orders of magnitude. There are far fewer long duration openings elicited by the semi-rigid agonists than by ACh at similar concentrations. The long duration full conductance never reached 1, nor was it ever 0.

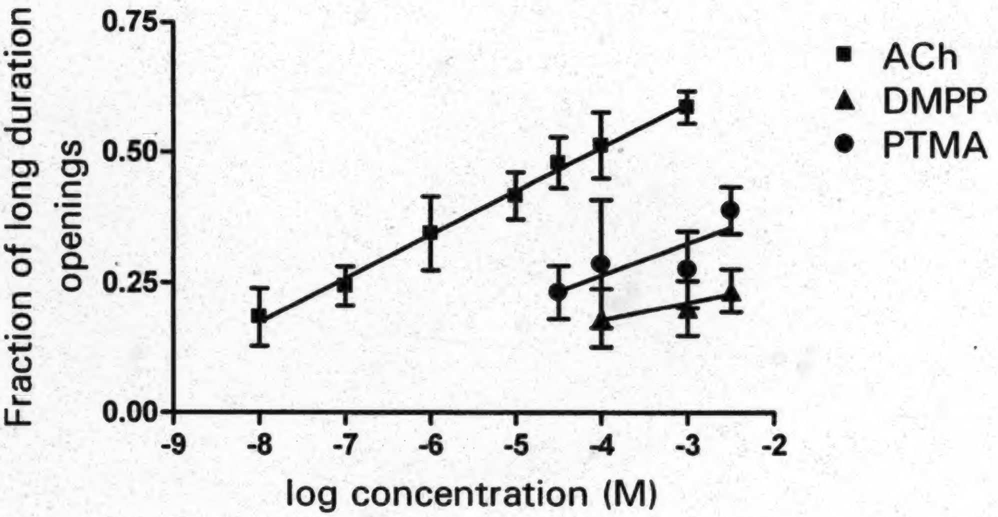
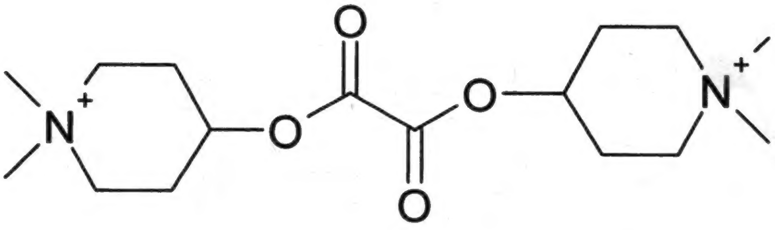

Fraction of long duration openings
0.75
0.50
0.25
0.00
-9
-8
-7
-6
-5
-4
-3
-2
log concentration (M)
■ ACh
▲ DMPP
● PTMA

Figure 3-10

Chemical structure of the novel semi-rigid agonist bis(N,N,-dimethylpiperidinyl) oxalate (DK1).



N+
O
O
O
O
N+

Figure 3-11

Sample single channel patch clamp recordings evoked by DK1. Currents were recorded at an electrode potential of +90 mV and filtered at 2 kHz. Upward deflections from baseline represent channel openings. All currents shown are subconductance currents (A) Currents elicited by 10 μM DK1. Few events during the 1 s recording period, all openings are short subconductance openings. (B) Currents evoked by 100 μM DK1. (C) Currents evoked by 1 mM DK1.

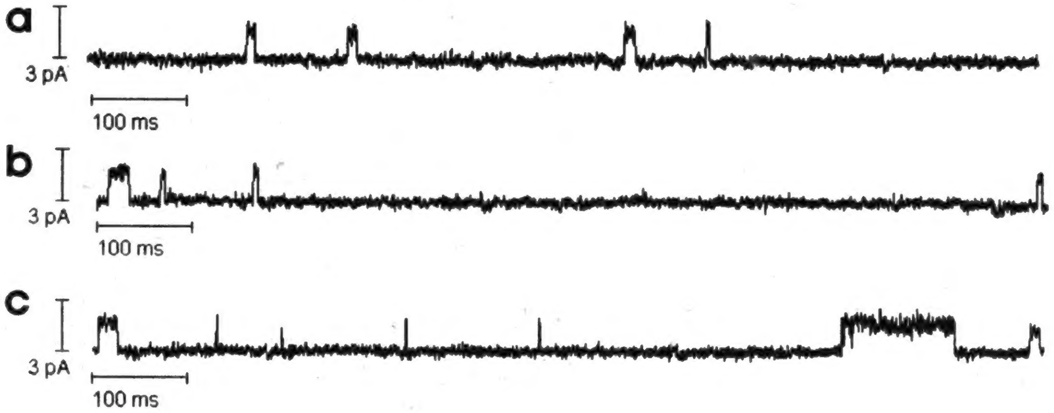

a
3 pA
100 ms
b
3 pA
100 ms
c
3 pA
100 ms

Figure 3-12

Sample amplitude histogram for currents evoked by 1 mM DK1 from a single inside-out patch. The mean amplitudes for the two open states are 1.72 ± 0.39 pA (s.d.) and 2.77 ± 0.64 pA (s.d.). The vast majority of events are subconductance openings (>97%).

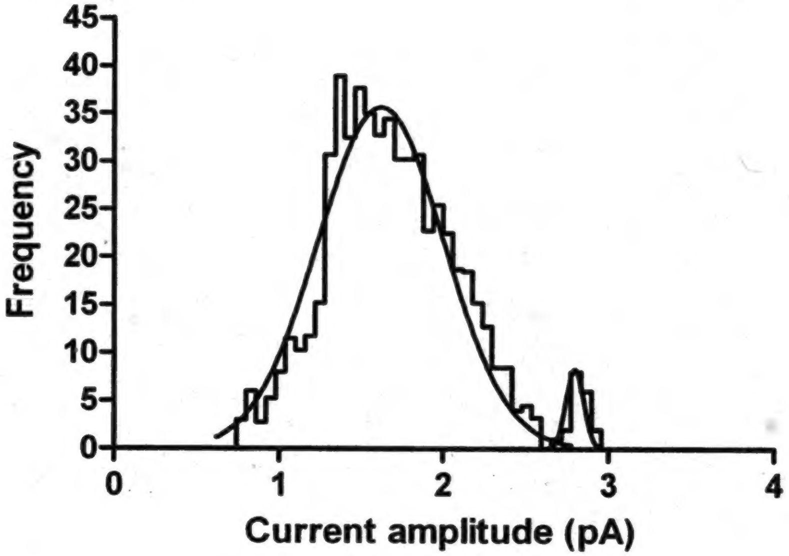

45
40
35
30
25
20
15
10
5
0
Frequency
0
1
2
3
4
Current amplitude (pA)

Figure 3-13

Full conductance currents as a fraction of all observed open events elicited by DK1. Few full conductance openings were recorded at all concentrations tested. The highest fraction of full conductance events, observed at a concentration of 3 mM, was 10% which was ~80% lower than that recorded for 1 mM ACh. Even at a very high concentration of DK1, few full conductance events are elicited.

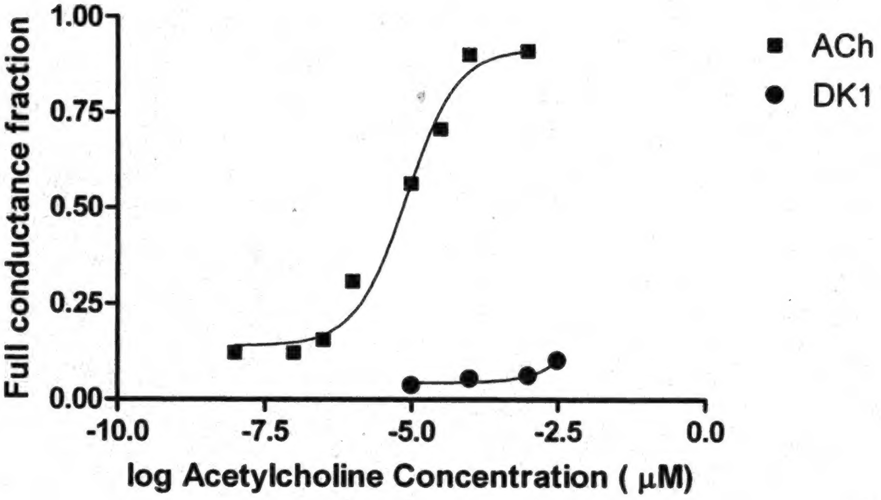

1.00
0.75
0.50
0.25
0.00
Full conductance fraction
-10.0
-7.5
-5.0
-2.5
0.0
log Acetylcholine Concentration (μM)
■ ACh
● DK1

Figure 3-14

Open duration analysis of subconductance currents evoked by DK1. All Sigworth-Sine plots are constructed from a single patch. (A) Sigworth-Sine plot of subconductance currents evoked by 10 μM DK1. Two exponential populations are not immediately evident, however analysis at higher concentrations revealed their presence. The shorter time constant was τ_S=0.36 ± 0.05 ms and the longer time constant was τ_L=7.03 ± 2.48 ms. (B) Sigworth-Sine plot of subconductance currents evoked by 1 mM DK1. Similar time constants were found at this higher concentration, where τ_S=0.46 ± 0.06 ms and τ_L=5.95 ± 1.62 ms.

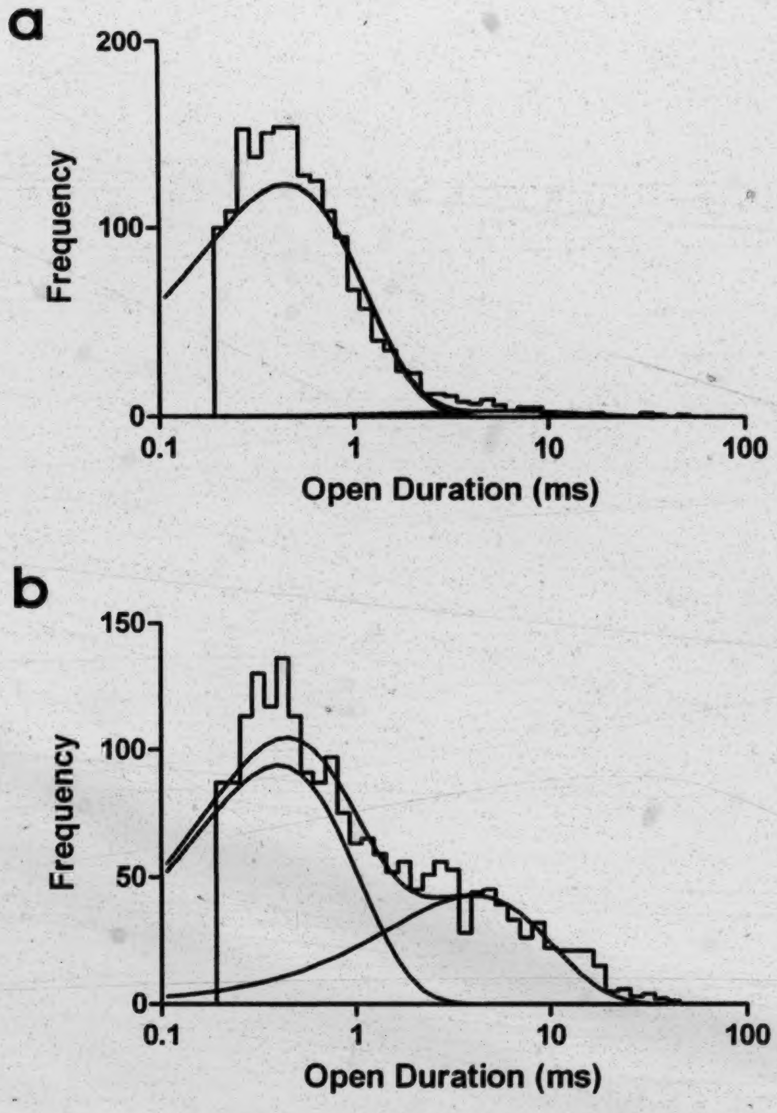

a
200
100
0
Frequency
0.1
1
10
100
Open Duration (ms)
b
150
100
50
0
Frequency
0.1
1
10
100
Open Duration (ms)

Figure 3-15

Stability of full conductance time constants elicited by DK1. There is no significant difference ($P>0.05$) between the time constants measured over the entire concentration range for both the shorter (τ_S) and longer (τ_L) time constants.

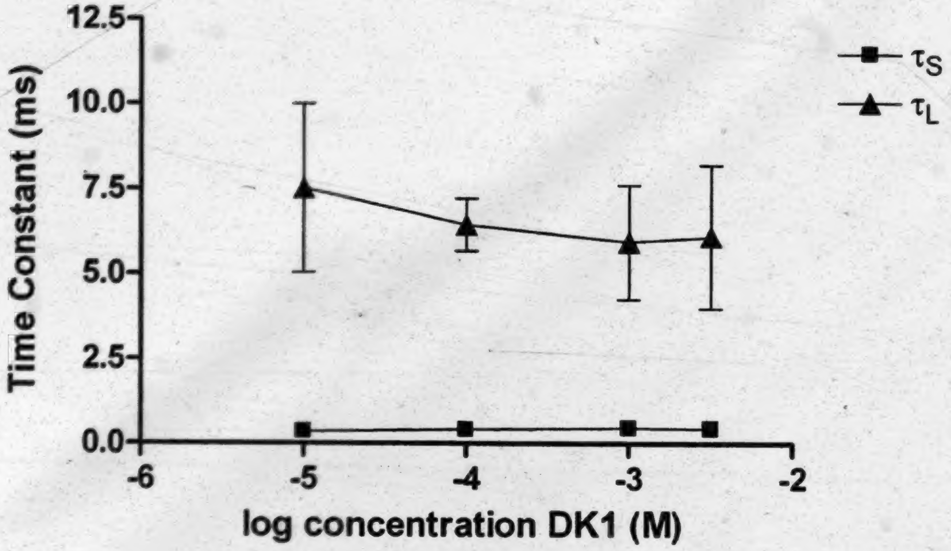
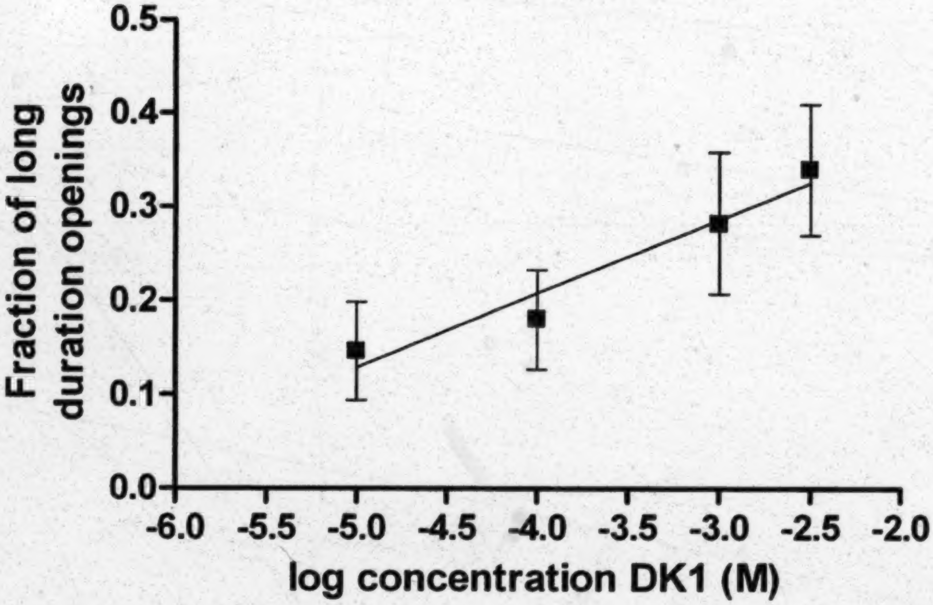

12.5
10.0
7.5
5.0
2.5
0.0
Time Constant (ms)
-6
-5
-4
-3
-2
log concentration DK1 (M)
τS
τL

Figure 3-16

Fraction of the total area contributed by long duration subconductance openings elicited by DK1. Although there was no significant concentration-dependent increase in the proportion of longer duration (τ_L) subconductance openings, there appears to be a trend toward an ever increasing proportion of long duration openings. If higher concentrations of DK1 could have been tested, it is quite possible that a statistically significant increase in the fraction of long duration openings with concentration would have been observed.


Fraction of long duration openings
0.5
0.4
0.3
0.2
0.1
0.0
-6.0
-5.5
-5.0
-4.5
-4.0
-3.5
-3.0
-2.5
-2.0
log concentration DK1 (M)

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CHAPTER 4

General discussion and conclusions

GENERAL DISCUSSION

A number of experimental systems have been used to study the single channel activity of the nicotinic acetylcholine receptor. Early single channel studies used muscle fibres dissected from either frog or mouse as these fibres contained high concentrations of receptor and were relatively easy to prepare (Colquhoun & Sakmann, 1985; Hamill & Sakmann, 1981; Neher & Sakmann, 1976). Shortly after these early reports, a mouse clonal muscle cell line (BC3H1) (Schubert *et al.*, 1974) came into use for single channel patch clamp studies (Sine & Steinbach, 1984a; Sine & Steinbach, 1984b; Wachtel, 1987). This cell line eliminated the need to continuously dissect out fresh muscle fibres which could not be used for long periods of time. Around the same time, biochemical methods for the extraction of nAChRs from *Torpedo* electroplax and their reconstitution into giant liposomes were developed (Elliott *et al.*, 1980; Lindstrom *et al.*, 1980; Tank *et al.*, 1983). These developments enabled the study of the well-characterized *Torpedo* nAChR in a system which eliminated many of the variables inherent in cells. With the advent of heterologous expression systems such as *Xenopus* oocytes or HEK293 cells, the systems previously described have fallen into disfavour. The relative ease with which receptors can be expressed and mutations of the protein introduced has all but eliminated the use of muscle fibre preparations, cell lines, and giant liposomes.

The study of native nAChR reconstituted into giant liposomes presents a system in which many of the variables likely to affect channel activity are removed. Since the liposome contains no endogenous enzymes, it is highly unlikely that the receptor under investigation could be altered via phosphorylation to affect single channel kinetics, especially the rate of desensitization (Huganir *et al.*, 1986; Ochoa *et al.*, 1989). Furthermore, a membrane preparation that has been purified represents a system in which few other channels are likely to be present. Many expression systems contain numerous endogenous channels which may affect the quality of single channel recordings. *Xenopus* oocytes, for example, contain a large number of stretch-activated cation-selective channels (Methfessel *et al.*, 1986; Yang & Sachs, 1990). Other endogenous channels reported to exist in *Xenopus* oocytes include chloride (Tokimasa & North, 1996; Weber *et al.*, 1995), sodium (Krafte & Volberg, 1992; Methfessel *et al.*, 1986), and potassium channels (Bauer *et al.*, 1996; Krause *et al.*, 1996). Furthermore, oocytes contain muscarinic acetylcholine receptors (Barnard *et al.*, 1982) which, in turn, may affect the kinetics of a LGIC via the activation of at least one of a variety of protein kinases. The fact that there may be a number of different channel types present in any given patch limits the ability to analyze single channel currents electronically, especially from channels that exhibit subconductance openings.

Although nicotinic receptors are reconstituted into giant liposomes from a highly enriched source of receptor protein within the *Torpedo* electroplax, there are still some other channel types that are nevertheless incorporated into these preparations. A voltage-gated chloride channel that is commonly found on the non-innervated face of the electroplax has been reported in membrane preparations reconstituted into giant liposomes (Goldberg & Miller, 1991; Tank *et al.*, 1982). Furthermore, a voltage-gated potassium channel has been reported to exist in the *Torpedo* electroplax (Edry-Schiller *et al.*, 1991). These channels, although likely present in the membrane preparations, constitute a very small fraction of all channels present as the concentration of nicotinic receptors found in the electroplax is extremely high. Furthermore, as these other channels are voltage-gated, the use of a hyperpolarized holding potential should decrease the likelihood that these channels will enter the open state. Their presence was not found to be troublesome in the present work and these other channels were easily distinguishable from nAChR channel openings by amplitude and/or the direction of current flow.

Receptors expressed or reconstituted into systems that contain lipid environments different from that experienced by the receptor *in vivo*, may elicit non-physiological responses. It has been shown that the lipid environment surrounding the receptor modulates the kinetics of the receptor (Chang *et al.*, 1995; Sunshine & McNamee, 1992) (Criado *et al.*, 1984; Zanello *et al.*, 1996). Liposomes created for this thesis contained only one lipid, L-phosphatidylcholine. As native membranes would contain other lipids such as cholesterol, phosphatidylserine, and phosphatidylethanolamine, the artificial bilayers of liposomes are not a physiological representation of the lipid environment and this may affect the single channel currents of the receptor. Furthermore, the absence of cholesterol affects the activation of the receptor as it has been reported that cholesterol is required to stabilize the conformation changes required to enter the open state (Addona *et al.*, 1998; Baenziger *et al.*, 2000). Many of these problems were avoided, however, as crude membranes were reconstituted into the giant liposomes, therefore the receptors would have been surrounded by their native lipid environment.

Nicotinic acetylcholine receptors reconstituted into giant liposomes provide a system in which many of the variables present in living cell expression systems are removed. However this system is not without its own problems. One major drawback to this system is the inability to use giant liposomes for whole cell recordings. Generally, cell expression systems allow the characterization of both whole cell and single channel currents, thus permitting a greater understanding of the kinetic of channel activation within the same model system. Giant liposomes, on the other hand, are incapable of being used for whole cell recordings as the membranes lack a cytoskeleton and therefore any rupture in the membrane leads to its collapse. Furthermore, if one is sufficiently careful to break the membrane without completely destroying the liposome, the lipids surrounding the site of breakage rapidly reform and thus access to the inside of the liposome is lost. Since whole cell recordings cannot be obtained from giant liposomes, such data must be collected from a separate expression system. It becomes difficult to account adequately for all variables between the two systems and extrapolation of the data from one system to the other must contain caveats.

The occurrence of subconductance currents have been reported in some *Torpedo* nAChR preparations (Riquelme *et al.*, 1990; Tank *et al.*, 1983) and not in others (Labarca *et al.*, 1984; Labarca *et al.*, 1985). It has been suggested that different methods used to extract the receptor from the *Torpedo* electroplax may explain these differences (Steinbach, 1989). The use of detergents to solubilize the receptor may induce a conformational looseness that results in the receptor not being able to efficiently enter the full conductance state (Sachs, 1983). If this hypothesis is correct, it may be that certain extraction protocols increase the likelihood that the receptor will enter the subconductance state due to changes in the electrostatic interactions within the receptor protein. This does not mean, however, that the receptors from these extraction protocols produce a non-physiological activation of the receptor, as subconductance currents have been reported in both cultured cells that natively express nAChRs and primary tissues (Auerbach & Sachs, 1983; Auerbach & Sachs, 1984; Hamill & Sakmann, 1981). Since the subconductance state has been reported in native nicotinic receptors, it may possible that the extraction and reconstitution protocol used by those who do not observe subconductance currents may alter the receptor in such a way that is it unlikely that the receptor can open to the subconductance state. Alternatively, the brevity of subconductance currents may lead to their being overlooked, or ignored by some authors.

It is important to elucidate all the states of the receptor accurately so that the sequence events from agonist binding to the opening of the channel gate can be fully understood. The charge carried by a subconductance opening is very small relative to a full conductance opening as subconductance currents are not only smaller in amplitude, but also shorter in duration. Physiologically, therefore, it is the full conductance that plays the major role in fast synaptic transmission. Although the charge carried by the subconductance state is small, it may play a role in synaptogenesis. Low basal concentrations of ACh present within the neuromuscular junction as a result of the non-quantal release of ACh will result in some activation of the receptor. At such concentrations, the predominant state will be the subconductance state. It has been shown that activation of the nAChR and the resulting calcium influx can repress AChR gene transcription (Huang *et al.*, 1994; Adams and Goldman, 1998). A small charge transfer, therefore, from subconductance openings can be argued to have an influence on gene transcription of the AChR.

CONCLUSIONS

Nature of subconductance currents

The debate over the nature of subconductance currents can be divided into two camps: those that believe they result from a partial occlusion of the channel pore (Strecker & Jackson, 1989; Takeda & Trautmann, 1984) and those that believe subconductance openings are a manifestation of separate conformational states of the receptor (Auerbach & Sachs, 1983; Auerbach & Sachs, 1984; Hamill & Sakmann, 1981; Morris & Montpetit, 1986).

From our data, it is apparent that the partial occlusion hypothesis cannot be supported. This hypothesis proposes that the occupation of a binding site by a ligand within the ion permeation pathway itself increases the energy barrier encountered by cations and thus decreases the flow of ions through the channel. According to this hypothesis it would be expected that an increase in the concentration of agonist would result in a higher probability of partially occluding the channel pore as has been demonstrated for classical full channel block (Neher & Steinbach, 1978; Ogden *et al.*, 1981; Ogden & Colquhoun, 1985). In contrast, we found that the occurrence of subconductance currents bears an inverse relationship to agonist concentration. The highest concentrations of agonists produced the lowest proportion of subconductance openings. When the frequency of subconductance currents was examined within a defined time period, we found that the rate of subconductance events increased slightly over a concentration range of 1-30 μM but reached a plateau rapidly thereafter. Thus, although the proportion of openings contributed by subconductance openings diminished with concentration as the frequency of full conductance openings increased, the absolute number of subconductance openings remained stable. Furthermore, we found that the time constants of all open states remained stable across the concentration, indicating that there was minimal channel block of the receptor. This is not to be expected from a channel block mechanism which would predict an increase in subconductance currents with concentration.

If the lower conductance of the receptor is the result of the partial occlusion of the channel due to binding of the agonist to a site within the pore, one would also predict that larger molecules would produce a greater restriction to the flow of ions than smaller molecules. When we examine the subconductance currents evoked by a variety of agonists, we find that the amplitude of the subconductance is constant despite a wide variance in charge distribution and stearic hindrance. Work not included in this thesis but using a variety of other agonists suggests that the size and structure of the agonist has no effect on the amplitude of the subconductance current. The smallest agonist that was investigated but not included in this thesis, tetramethylammonium, elicited subconductance currents with the same amplitude as larger molecules such as suberyldicholine and lobeline. These findings are in agreement with those reported by Morris and Montpetit (1986) and suggest that these agonists do not bind within a site of the channel lumen to directly obstruct the flow of ions.

To investigate the two hypotheses further, it may be useful to perform a series of voltage experiments at different holding potentials to determine if the subconductance state exhibits any voltage-dependence. Contradictory evidence exists, with some groups reporting that there is a weak association between holding potential and the frequency of subconductance currents (Strecker & Jackson, 1989; Takeda & Trautmann, 1984), whereas others report that there is no such association (Morris *et al.*, 1989; Morris & Montpetit, 1986). Varied holding potentials were not used in this thesis as this was not the primary focus of the inquiry. However, the elucidation of the effect of voltage on the subconductance may provide a clearer picture of the nature of the subconductance state.

Different sites responsible for the activation of the subconductance and full conductance states

From the previous considerations, it is concluded that the subconductance state is an independent entity and results from a distinct conformational state of the receptor. For the receptor to enter this state, we hypothesize that an agonist must occupy a site or sites independent from the site(s) responsible for the full conductance state based on the observation of the relationship between the EC_{50} for full and subconductance currents using different agonists. Evidence suggests that there may exist distinct agonist binding sites with separate affinities for agonist on the nAChR (Dunn *et al.*, 1980; Dunn *et al.*, 1983; Dunn & Raftery, 1982a; Dunn & Raftery, 1982b). Furthermore, there may exist two subsites within each high affinity binding site which can be bridged by a bis-functional agonist such as suberyldicholine (Dunn & Raftery, 1997a; Dunn & Raftery, 1997b).

It was clear from the activation of the receptor by ACh that subconductance currents were more frequent than full conductance openings at low concentrations. In fact, the majority of events were subconductance openings up to a concentration of 5 μM. The proportion of full conductance currents increased in a concentration-dependent manner, reaching a plateau at 1 mM. These data suggested that the progression from the subconductance to the full conductance state required a greater occupancy of the receptor, although it was unclear as to whether the sites were independent of one another or linked (discussed later).

When the semi-rigid, poor partial agonists, DMPP and PTMA were examined, a similar pattern emerged. These agonists required much higher concentrations to reach the same proportions of full conductance events as that observed with ACh. Furthermore, within the technical limitations of the system, DMPP and PTMA did not reach a maximal plateau comparable to that of ACh. The original impetus to study these agonists was provided by an earlier study by Kawai *et al.* (2000). The authors found that the semi-rigid agonist methylarecolone elicited almost exclusively subconductance currents, which was in contrast to the more traditional agonist carbamylcholine that elicited a majority of full conductance currents. It was hypothesized that the semi-rigid structure of methylarecolone prevented the receptor from adopting the conformation necessary to allow the maximal flow of ions through the channel. It is clear from the present data, however, that the structure of these compounds does not preclude the adoption of the full conductance conformation. As the study by Kawai *et al.* (2000) examined only one concentration of methylarecolone (2 μM), it was impossible to achieve the full occupancy of the receptor as much higher concentrations would be required. From these data, it can be concluded that the sites responsible for full conductance openings are agonist-sensitive and that as a much higher concentration of semi-rigid agonist is required to activate the full conductance state, the corollary would seem to be that these sites bind semi-rigid agonists with much lower affinity.

We would agree that there exists more than one site responsible for the modulation of the full conductance state from the existence of two distinct populations of full conductance open durations as has been previously reported (Labarca *et al.*, 1984; Labarca *et al.*, 1985). At low concentrations of agonist, short open duration full conductance events predominate. As the concentration increases, the proportion of longer duration openings increases in a linear fashion. This is true of all agonists tested, although the semi-rigid agonists required much higher concentrations to produce the same proportion of long duration openings as ACh. The concentration-dependent increase of the population of longer duration full conductance openings confirms previous reports (Labarca *et al.*, 1984; Labarca *et al.*, 1985) that there are multiple sites located on the receptor that modulate the full conductance state.

Kinetic scheme for the activation of the receptor

The original two-state kinetic model proposed by Monod, Wyman and Changeux (1965) has served as the basis of many kinetic schemes derived from single channel recordings (Akk *et al.*, 1996; Auerbach *et al.*, 1996; Colquhoun *et al.*, 2003). This evidence greatly complicates the matter of modeling the activation of the nAChR using single channel kinetics.

The existence of the subconductance state has long been acknowledged but there has not been an effort to incorporate this state into the existing kinetic schemes. With the knowledge that there may exist a number of agonist binding sites on the receptor and on the basis that a separation of concentration-effect curves for subconductance and full conductance currents occured when a semi-rigid agonist is substituted for ACh, we hypothesized that the subconductance state arose from the occupation of a site separate from the site(s) that lead to the full conductance activation of the receptor.

Constructing a kinetic scheme for the activation of the nAChR which accounts for both subconductance and full conductance currents within a single, sequential, scheme is extremely difficult. Subconductance currents apparently occur independently of full conductance openings and the incidence of Type II and Type III subconductances are so low as to be purely a coincidence of the subconductance and full conductance state. There is no empirical evidence for a sequential connection between the two. Thus, the receptor is not required to enter the subconductance state before entering the full conductance state. Therefore, the kinetics of the subconductance state should be treated completely independently of any kinetic scheme involving the full conductance state.

Two open subconductance states

The subconductance state consists of a single open duration when ACh, DMPP, or PTMA is used. Subconductance currents elicited by the novel agonist bis(N,N,-dimethylpiperidinyl) oxalate (DK1) reveals two distinct open durations similar to those observed for full conductance openings. Two possibilities exist to explain this discrepancy between these agonists. One potential explanation is that the bisfunctional structure of DK1 may adopt multiple conformations within the binding pocket of the receptor, stabilizing the open state of the receptor to different degrees, thus resulting in two different populations of subconductance open durations. The other possibility is that the other agonists (ACh, DMPP, and PTMA) do indeed elicit two distinct subconductance open durations. This shorter duration opening is so short that it falls below the threshold filter frequency and is eliminated from the recordings. If this second hypothesis is correct, the concentration-dependent increase in the frequency of longer duration subconductance openings suggests that there may exist two sites responsible for subconductance activate that operate in a manner similar to the full conductance activation sites. The occupation of one site would result in the channel opening to the short duration subconductance state, whereas the occupation of both sites would elicit the longer duration subconductance open state.

It is impossible to determine which of the two hypotheses are correct and further study is required. Conducting experiments at lower temperatures (8-10°C) may increase the subconductance open durations enough to be adequately resolved. The synthesis of compounds similar to DK1 may also provide insight in its mechanism, although the addition of intervening methyl groups resulted in the loss of all activity. Perhaps compounds with a similar interonium distance may be synthesized which activate the receptor and which will allow the further investigation of this phenomenon.

Future directions and conclusions

The location of the site(s) responsible for subconductance currents is, as yet, unknown. Inactivation of the two high affinity binding sites via reduction and alkylation with DTT still resulted in subconductance openings, indicating a separate mechanism for the generation of the subconductance state (Strecker & Jackson, 1989). Future characterization of the location of the subconductance site(s) may be done by using a class of compounds known as bischolines. These molecules consist of two quaternary nitrogen groups separated by a known number of methyl groups. Evidence from fluorescence and radioligand binding suggests that these compounds, if they possess a chain length of ~15Å, may be able to bridge the two subsites within the high affinity binding pocket (Dunn & Raftery, 1997a; Dunn & Raftery, 1997b). Further evidence suggests that Trp-86 on the α-subunit may form part of the subsite, as it is predicted to lie

15-20Å from the high affinity binding site (Kapur *et al.*, 2006). Mutation of this amino acid resulted in no significant difference between the concentration-effect curves of the mutant and wild type receptors, whereas the mutation resulted in a ~500-fold increase in the EC50 for suberyldicholine. Preliminary evidence from my experiments suggests that at the single channel level, bischolines with chain lengths greater than 13Å elicit mainly full conductance openings, whereas those bischolines with chain lengths less than 13Å elicit channel activation in a manner similar to ACh. A more in depth analysis of these findings is warranted, with particular attention paid to open duration analysis. Focusing on duration analysis may reveal what role, if any, these subsites play in determining the open state of the receptor.

The role of desensitization was not investigated in this thesis as it greatly complicates single channel analysis. This state is, however, important in the kinetic scheme of receptor activation and should be addressed. Of particular interest for future investigation is the effect of the occupancy of the subconductance site(s) on the desensitization rate of the receptor. Since it is very difficult to amass a large enough sample of receptors and their desensitization kinetics from single channel recordings, these experiments would have to be performed using the whole-cell recording technique. *Xenopus* oocytes injected with nAChR cDNA present the ideal experimental system as they express large quantities of receptor. By choosing a concentration of agonist that elicited mainly subconductance openings with few full conductance events, the likelihood of the full conductance activation sites being occupied is low and therefore the currents measured should be mainly subconductance openings. The partial agonists DMPP and

PTMA present ideal candidates for this study as there exists a significant separation of concentrations that activate subconductance and full conductance currents.

To conclude, the data presented here suggest that subconductance currents arise from an independent state of the receptor. This state is most likely the result of the occupancy of one or more binding sites which are independent of the sites responsible for the full conductance state. Subconductance currents, therefore, need to be recognized in any kinetic schemes that attempt to accurately model the activation of the nicotinic acetylcholine receptor.

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